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Estimation of succinic acid in biological materials. By KATHLEEN RODGERS

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The rate of incorporation of [$^{35}$S]methionine and [$^{35}$S]cystine into proteolipids and proteins of rat brain. By M. K. GAITONDE

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Formation of chromenols on alumina from quinones having an isoprenoid side chain. By F. W. Hemming, R. A. Morton and J. F. Pennock

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**Abbreviations.** These must not be used in the title, introductory section, headings or Summary. Names of compounds (except those listed below) should not be abbreviated unless they are unwieldy and have to be used very frequently. All abbreviations except those listed below must be defined at the beginning of the Experimental section and not, as previously, at the first mention. The following may be used without definition:

| | |
|---|---|
| ADP, CDP, GDP, IDP, UDP | 5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine and uridine |
| AMP etc. | Adenosine 5′-phosphate etc. |
| ATP etc. | Adenosine 5′-triphosphate etc. |
| CoA | Coenzyme A |
| DNA | Deoxyribonucleic acid |
| DPN | Diphosphopyridine nucleotide |
| EDTA | Ethylenediaminetetra-acetate |
| FMN | Flavin mononucleotide |
| FAD | Flavin–adenine dinucleotide |
| GSH, GSSG | Glutathione, reduced and oxidized |
| NAD | Nicotinamide–adenine dinucleotide |
| NADP | Nicotinamide–adenine dinucleotide phosphate |
| NMN | Nicotinamide mononucleotide |
| RNA | Ribonucleic acid |
| TPN | Triphosphopyridine nucleotide |
| tris | 2-Amino-2-hydroxymethylpropane-1:3-diol |

*Abbreviations for amino acids and carbohydrates.* Those marked * and † in *Suggestions to Authors* are to be used only for representation of polymers or sequences and in Tables and Figures.

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The abstract should outline as briefly as possible the results and definitive conclusions of the work submitted. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet in the following form: title; name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); abstract. For example:

> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland)—(Abstract).

**Form of papers submitted for publication.** Papers should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English. Footnotes should be avoided as far as possible.

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author, who should first consult the detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions* (obtainable from the Editorial Office, 133–135 Oxford Street, London, W. 1, price 1*s.* 6*d.*). Authors should also refer to a current issue of the *Journal* in order to make themselves familiar with the typographical conventions, use of cross-headings, lay-out of tables, citation of references, etc. The need for editorial revision of badly prepared typescript or diagrams will delay publication. Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the *Journal*. Sufficient information should be included to permit repetition of the experimental work.

Papers should be written concisely and, generally, should be divided clearly into sections as follows: (*a*) introduction, containing the reasons for doing the work; (*b*) Experimental methods: in chemical papers this section may appear towards the end; (*c*) Results: these should be given concisely; the use of both tables and figures to illustrate the same results will only rarely be permitted; (*d*) Discussion: the presentation of the results should be separated from the discussion of their significance; this section should be strictly limited to discussion, and should not recapitulate results; (*e*) a Summary, about 3% of the length of the paper: the paragraphs of the Summary should be numbered; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*b*)–(*d*) is not binding on authors; another arrangement may be more suitable.

*The Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

**Illustrations.** Diagrams that do not conform with the directions given in *Notes on Preparation of Illustrations* (obtainable from the Editorial Office) may have to be redrawn by the Press and the expense charged to the author. Legends and captions should be written so that the general meaning of each illustration can be understood without reference to the text, and so that the exact experimental conditions used to obtain the results illustrated are made clear. Illustrations requiring reproduction as half-tone plates should be avoided whenever possible. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published.

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**Tables.** Tables should have headings which make their general meaning comprehensible without reference to the text. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or ',,' are not to be used.

PAUL HAAS

*Biochem. J.* (1961) **80**, 1



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# Obituary Notice

## PAUL HAAS
## (1877–1960)

Paul Haas was born in London in 1877 and died at Cheam, Surrey, on 6 April 1960. His father was Professor of Sanskrit at University College and his mother was an accomplished pianist who had a European reputation as a specially gifted interpreter of Bach. Her son inherited her musical appreciation.

Paul attended University College School in its Gower Street days before entering the parent College as an undergraduate. Then, as always, University College was one of the most stimulating academic centres in the world. He was there in the days of Ramsay, Bayliss, Starling, Oliver and Flinders Petrie. After obtaining the B.Sc. degree Haas went to Geneva, where he worked with Carl Graebe, who is well known for his synthesis of alizarin, carried out with Liebermann. Haas prepared the methyl and the phenyl ketones of acenaphthene and studied their oxidation to carboxyacenaphthenes. By the Beckmann transformation of the oximes he finally obtained 4-aminoacenaphthene, thus establishing that Friedel–Crafts substitution occurs at position 4. This was checked by conversion of the phenyl ketone into benzoylnaphthalic acid. This, on loss of carbon dioxide, gave α-naphthyl phenyl ketone and finally α-naphthylamine. His results were presented in July 1901 at the University of Freiburg im Breisgau as a thesis for the degree of Ph.D., the senior examiner being L. Gattermann. Haas did not, however, publish any research work from Freiburg. It does not appear that his time in Geneva influenced his choice of a theme for future work, and fourteen years were to elapse before he began his researches on marine algae. During that time he enlarged his experience in organic chemistry and acquired that skill in following up unusual or unexplained results which characterized so much of his later work.

His first post was with A. W. Crossley in the Chemistry Department of St Thomas's Hospital Medical School. Crossley had worked with Emil Fischer at Würzburg on the oxidation of saccharic acid and mucic acid, but on returning to England he began a long series of researches on 1:1-dimethyl-*cyclo*hexane-3:5-dione (dimedone). Haas associated himself with this work and described the reaction of dimedone and of the corresponding 5-chloro compound (obtained by the action of phosphorus trichloride) on ammonia and primary amines. With dimedone one or two molecules of water can be eliminated. The chloro derivative reacts with $2\ R{\cdot}NH_2$. When one amine residue is introduced the remaining oxygen is enolic, but is ketonic when the amine residue is acetylated. Another paper appeared a few months later, and from the superscription (degrees were appended to authors' names in those days) it appears that Haas had obtained his D.Sc. in the interval. One molecule of *m*- or *p*-phenylenediamine reacts with either (*a*) one molecule or (*b*) two molecules of dimedone. The relations of the two products (A) and (B) to each other were studied. Acetylation of (A) occurs on the –NHR group and not on the hydroxyl group. The chloro derivative reacts with two molecules of diamine, giving a compound (C) containing no oxygen.

On analysis of many compounds of these types Haas obtained low figures for carbon and hydrogen and a high nitrogen value when a copper oxide tube and a copper spiral were used. The hydrochlorides of the bases, however, gave normal values. Dunstan and Carr in 1896 had made an exactly similar observation with aconitine and its hydrochloride, which they attributed to formation of methane. Admixture of the aconitine with cuprous chloride gave correct figures. Somewhat analogous results are described in *J. chem. Soc.* (1923, **123**, 1050). Analyses by Haas of the evolved 'nitrogen' confirmed the formation of methane. By mixing his compounds with cuprous, cupric or lead chloride, and by using a tube containing lead chromate with a silver spiral, correct results were obtained. Traces of chlorine appeared to suffice in some cases. Several compounds gave normal results for nitrogen because the copper oxide tube had been used for the combustion of a hydrochloride. Haas showed that, on passage of methane with carbon dioxide through a copper oxide tube, up to 43% was unburned; when he used the oxide and cuprous chloride combustion was complete. Methane formation was attributed by Haas to the presence of the hydroaromatic ring; with compounds of type B containing two such rings, the abnormal results were more pronounced. He considered that the *gem*-dimethyl group might be concerned, as the compound from 1-methyl*cyclo*hexane-3:5-dione and *m*-phenylenediamine gave a correct result for nitrogen in a tube containing only copper oxide. Other monomethyl compounds of this type were not tested.

In 1912 Haas published some work on reactions of hippuric acid. This compound has always had an attraction for students of science and medicine as it represents the first established case of detoxication by glycine. R. T. Williams in *Detoxication Mechanisms* (Chapman and Hall, 1959, pp. 13–14),

states that it was Keller in 1842 who, in Wöhler's laboratory in Göttingen, definitely established that benzoic acid and glycine were concerned in the biological formation of hippuric acid. It is therefore not surprising that Haas, familiar with this acid through the instruction of students in the laboratory, should examine critically any published work on its derivatives. Drechsel had obtained, on benzoylation of the products from the acid hydrolysis of casein, a substance believed to be α-aminohippuric acid (2-amino-2-benzamidoacetic acid) and which he stated to yield diaminoacetic acid on hydrolysis. Other workers found difficulty in preparing this second acid. From hippuric acid, bromine and red phosphorus Haas obtained, on treatment of the product with (*a*) water and (*b*) ammonia, α-hydroxyhippuric acid and either benzamide or the amide of α-hydroxyhippuric acid respectively. No α-aminohippuric acid was isolated. α-Hydroxyhippuric acid,

$$C_6H_5 \cdot CO \cdot NH \cdot CH(OH) \cdot CO_2H,$$

with acids or alkalis readily gives benzamide and glyoxylic acid, a reaction which is analogous to the fission of 'aldehyde-ammonias' by acid:

$$R \cdot CH(OH) \cdot NH_2 \rightarrow R \cdot CHO + NH_3.$$

This formation of glyoxylic acid on bromination and hydrolysis is a delicate test for hippuric acid. The reaction mixture when treated with sulphuric acid and a trace of protein gives the well-known purple colour.

Another paper again illustrates Haas's scientific curiosity and careful observation. α-Hydroxyhippuric acid exhibits a peculiar behaviour on heating. It melts and froths at about 150°, resolidifies and finally melts and decomposes about 210°. Haas identified the products of this reaction, in which dibenzamidoacetic acid is formed. This, on acid hydrolysis, gave as primary products glyoxylic acid and benzamide. Drechsel's diaminoacetic acid was not obtained.

In 1913 he joined the Department of Botany at University College, where he was associated for many years in teaching, authorship and research with T. G. Hill, then Reader in Vegetable Physiology and later Professor of Plant Physiology. Hill had also been on the staff at St Thomas's. Haas's introduction to marine algae arose from the shortage of gelatin in 1915, and Professor Oliver suggested that Haas and Hill should study carrageen or Irish moss (*Chondrus crispus*) with a view to its use as a substitute. The ready gelatinization of aqueous extracts of the alga was well known and had already found some technical applications. In 1921 Haas announced in *The Biochemical Journal* that carrageen contained 'ethereal sulphates' in the form of salts, chiefly of calcium, and that the $-O \cdot SO_2 \cdot O-$ group was linked to carbohydrate. Compounds of this type were already known in animals, but Haas remarked: 'the present instance would appear to be the first recorded case of the existence of carbohydrate esters of sulphuric acid among plant products'.

It was already known that the ash of carrageen is mainly calcium sulphate and that dialysis of aqueous extracts of the alga does not remove the calcium. Haas showed, however, that the sulphate ion is absent from such extracts but appears on hydrolysis with hydrochloric acid. The amount of sulphate then present in solution is twice as great as that contained in the ash, and he showed that carrageen on heating loses sulphuric acid. Later Haas and Miss Russell-Wells found that polysaccharide sulphates from certain other algae lost less sulphuric acid on heating owing to the simultaneous formation of a very basic ash. The occurrence of carbohydrate esters of sulphuric acid in plants is now so well recognized that this fundamental work of Haas tends to be overlooked. A few months later Neuberg and Ohle, in the *Biochemische Zeitschrift*, reported the presence of conjugated sulphuric acid, possibly linked to carbohydrate, in agar. They cited Haas's results and some early work of Tamman in 1882 on the occurrence of 'ätherschwefelsäuren' in green peas.

In 1925 Haas and Hill showed that on anaerobic extraction of *Mercurialis perennis* (dog's mercury) or *M. annua* with water the colourless solution rapidly turned blue in air and finally yellow. The unknown compounds involved were named hermidin, cyanohermidin and chrysohermidin. The volumes of oxygen used in these two stages are equal. The redox potential of the system was measured by R. K. Cannan. This was the first study of a plant redox system to be made in Great Britain by botanists. In 1936 Haas, Hill and Miss B. Russell-Wells briefly reported in a letter to *Nature* on the stable blue substance formed on drying young shoots of *M. perennis* in air. It is different from cyanohermidin, and on keeping or boiling with water forms a red product. From this, by chemical and physical methods, they separated a series of red glycosides containing nitrogen and sulphur. No further information has been published on these pigments.

This work was followed by a study of the hexahydric alcohols in marine algae. Mannitol had been isolated by Stenhouse in 1844 from brown seaweeds, but Haas and Hill established its presence in *Pelvetia* species along with a mannitan. This was isolated as a dibenzal derivative under conditions which appeared to exclude the possibility of its formation as an artifact from mannitol. They also obtained dulcitol from alcoholic extracts of the dried red alga *Bostrychia scorpioides* and oxidized it to mucic acid. The syrup from the separation of the dulcitol was treated with benzaldehyde and hydrochloric acid in the hope of obtaining a benzal-

dulcitol, but the crystalline product was found to be a mixture of the di- and tri-benzal compounds of sorbitol and to yield this hexitol on acid hydrolysis. The hexa-acetate of sorbitol was also obtained on acetylation of the original syrup. No dulcitol or sorbitol could be found in eight other members of the Rhodophyceae.

During an examination of the carbohydrate metabolism of brown seaweeds Haas found on testing concentrated extracts with Fehling's solution that a mauve tint developed. This recalled the biuret reaction and was shown to be due to an octapeptide which yielded glutamic acid hydrochloride on hydrolysis. Then followed in 1933–35 two papers on algal metabolism in the *Annals of Botany*. These display the intimate knowledge acquired during twenty years, they draw analogies with fungi and with land plants and afford much stimulus to further work. The authors made the tentative suggestion that the peptides of brown algae are intermediate products, arising from a disturbance of metabolism due either to desiccation on exposure to air, or to low illumination and consequent interference with photosynthesis and protein metabolism. Ten red algae which grow under conditions of minimum exposure to air were found to contain no peptides. Two red 'calcareous' algae, which are incrusted with calcium carbonate containing some magnesium carbonate and consequently suffer from lack of illumination, contained peptides. A third red alga growing under shaded conditions also contained a peptide. The amino acids produced on hydrolysis were identified. These experiments, while not conclusive, certainly afford a *prima facie* case for the authors' hypothesis.

Chemists in the textile industry will recall the interest aroused by alginic acid in 1942. In 1931 Miss G. M. Bird and Haas made a useful contribution to the early chemistry of this compound by showing that it gave mannuronic acid on hydrolysis and confirming the formation of mannosaccharic acid on oxidation. They also proved that fucoidin, which accompanies alginic acid in the cell wall of *Laminaria* species, is a polysaccharide sulphate. The ash of seaweeds had long been known to contain phosphates, but in 1935 Haas and Hill showed that in *Chondrus crispus*, *Polysiphonia fastigiata* and *Dilsea edulis* the whole of the phosphorus in dialysed aqueous extracts is present as organic phosphate and is liberated as orthophosphoric acid on hydrolysis.

In 1935 Haas studied the volatile, odorous compound evolved by the red seaweed *Polysiphonia fastigiata* on removal from its habitat. The alga is frequently found growing on *Ascophyllum nodosum* in rock pools at low-water mark. Early in 1935, after a meeting of the Council of the Chemical Society, Paul Haas, William Wardlaw and I met under the Piccadilly archway. Haas showed us a small bottle containing a colourless liquid. 'I wish', said he, undoing the stopper, 'that you would tell me what you think this smell is. It reminds me of a phosphorus compound or of butadiene'. We asked what the liquid was. 'Water', he said, 'through which air has been drawn after passing over a wet red seaweed'. Wardlaw and I recognized the odour as that of a dialkyl sulphide, probably dimethyl sulphide, because he and E. G. Cox had made X-ray examinations of its co-ordination compounds with platinum salts and I had encountered methylated alkyl sulphides during work on mould metabolism. Shortly afterwards, in a paper read before The Biochemical Society in Oxford, Haas announced its identification as dimethyl sulphide and its characterization as the dibromide, the mercurichloride and the platinochloride. With Haas's permission and lively interest Miss Margaret Simpson in Leeds was able to show in 1947 that the precursor of the dimethyl sulphide in *P. fastigiata* was *S*-dimethyl-$\beta$-propiothetin,

$$(CH_3)_2\overset{+}{S}\cdot CH_2\cdot CH_2\cdot CO\cdot \overset{-}{O},$$

to isolate the thetin as its hydrochloride, to characterize it amply and to show that its fission to the sulphide was almost certainly enzymic. Later Cantoni furnished complete proof of this and studied the enzyme. In July 1947 du Vigneaud, at a lecture in London, recalled his earlier demonstration that *S*-dimethylacetothetin,

$$(CH_3)_2\overset{+}{S}\cdot CH_2\cdot CO\cdot \overset{-}{O},$$

was a methyl donor and supported the growth of young white rats on a diet containing homocystine but not methionine. He remarked that one would expect so excellent a methyl donor to occur in Nature. When a preliminary account of Miss Simpson's work appeared, G. A. Maw in du Vigneaud's laboratory tested the seaweed thetin and found that it also was a powerful methyl donor in white rats under the same dietary conditions. Dubnoff and Borsook then showed that the two thetin hydrochlorides could methylate homocysteine to methionine in liver or kidney preparations from rats. Neither thetin has been found in animals, nor has dimethylacetothetin been detected in Nature. The transmethylation observed by the American workers may possibly be due to the action of betaine transmethylase, which, Maw has suggested, may be identical with the thetin transmethylase of Dubnoff and Borsook. These results, followed by Cantoni's striking discovery of active methionine—the *S*-adenosinylmethioninesulphonium ion, also a thetin—greatly stimulated interest in natural sulphonium compounds.

In 1910 Haas published *Laboratory Notes on Organic Chemistry for Medical Students*, most of which could be used to-day. Three years later the first volume of Haas and Hill's *The Chemistry of*

*Plant Products* appeared, followed by Volume II in 1922. In an obituary notice of Hill in *Nature* (1956) we read: 'Volume II on Metabolic Processes can still be read by students with profit for its dynamic method of approach and presentation'. These three volumes were almost unique at the time they appeared and only considerations of space preclude further comment.

Haas had a flair for languages which was probably inherited from his father, and a particular affection for France, its language and its people. His German was fluent, and he also had a working knowledge of other European languages and taught himself Russian. It is not surprising that he was very interested in foreign students, who must have found him particularly sympathetic and a clear teacher. He never lost his tremendous desire to learn what people did, how things were done and how they worked. These things were an endless source of interest to him. The sea greatly attracted him and he made many trips on cargo boats, serving as one of the crew. He was an expert swimmer and a keen walker.

At the age of 61 he married Miss Beryl Grace Wood, a biologist whom he met while inspecting schools for the University of London. Their son, in a most happy continuity, is a student at St Thomas's Hospital Medical School, for which his father had a great affection that was also shared by Hill. Haas had a tall and dignified presence; his kindly courtesy recalled the manners of earlier and more spacious days. He was perhaps a little shy in the best sense of the term, and had that intangible, but true, humility which Dr C. H. Newman once so happily associated with the late R. H. A. Plimmer. The devotion with which he pursued, steadily and quietly, his chosen research, and his generous appreciation of the work of others, endeared him to his friends. He believed in personal work at the laboratory bench and, throughout his life, apart from T. G. Hill and Dr Barbara Russell-Wells, who were concerned in nearly all his biochemical work, he had only three or four collaborators.

Haas was a Fellow of University College, an Original Member of The Biochemical Society and a Fellow of the Chemical Society.

A colleague has sent me an appreciation of Haas's work from a somewhat unusual angle.

'Throughout the war of 1939–1945 he worked for the Inter-Services Research Bureau. This necessitated a very considerable change in his way of life but he adapted himself to it extraordinarily well; his natural charm of manner and his kindly benevolence towards his juniors secured for him a warm regard in the very mixed group of serving officers, scientists and engineers, young and old, among whom he worked. Although the problems with which he was concerned, nearly always unusual and often very urgently requiring a solution, were remote from normal University research, Haas always maintained the highest academic standard. He worked steadily, with constant attention to detail, and he never considered a problem as solved until he had applied to the solution the most exacting tests. On several occasions he showed great courage in experimenting with dangerous material which he felt he could not recommend for use until he had himself tested it. His colleagues will remember him for his absolute integrity and his stubbornness when he felt he was right: once he refused, point-blank, to give, to the high-ranking administrator who controlled his laboratory, information about the composition of an important material on the grounds that it was a scientific and technical matter and that security required that as few people as possible should know about it. On the other hand, he never made unreasonable demands on the administration; moreover, when his scientific colleagues were in need, his extensive knowledge of the by-ways of science was put freely at their disposal and he could nearly always make a suggestion which helped towards the solution of their problems. He was a fine scientist—conscientious, individual and dedicated.'

Dr Barbara Russell-Wells, who was associated with Haas in teaching and research in the Department of Botany at University College London for nearly 20 years, writes:

'it would be impossible to find a pleasanter or more loyal colleague than he was. I think that as both teacher and research worker his outstanding qualities were enthusiasm and patience. As a teacher he took a personal interest in each of his pupils and was always eminently approachable. He encouraged them to discuss their difficulties with him and was never too busy to listen to them. He took endless trouble in preparing demonstrations for practical classes and never set an experiment which we had not tried out first. I think all his students regarded him as a friend as well as a teacher.

Each year he gave a course of elementary lectures on economic botany including such subjects as tobacco, tea, sugar, paper, rubber and so on. For each one he obtained an enormous number of specimens for each stage from the raw material to the finished product and, where possible, showed experiments illustrating the various processes of manufacture. He took great pains to make his lectures interesting and living.

His enthusiasm for research amounted to a passion. He could become very depressed when things went wrong and correspondingly excited when they went right. I clearly remember his delight when final proof was obtained of the existence of polysaccharide sulphates in the seaweeds.

He was extremely modest and most generous in sharing the credit for his own achievements with his colleagues. He hated injustice and would be most indignant if he thought that any of his colleagues or students had been unfairly treated in any way. In fact, he was a most lovable man, with many friends, who always seemed to bring out the best in the people with whom he came in contact.'

To extend and impart knowledge has been one of the greatest privileges of disciplined minds throughout the ages. It may truly be said of Haas:

'—and gladly wolde he lerne, and gladly teche.'

FREDERICK CHALLENGER

*Biochem. J.* (1961) **80**, 5



# Studies on Alkaline Phosphatases

## 3. INFLUENCE OF AGE OF FOWL AND MAMMALS ON pH OPTIMA*

BY I. MOTZOK AND H. D. BRANION
*Department of Nutrition, Ontario Agricultural College, Guelph, Canada*

(*Received* 7 *November* 1960)

The pH required for optimum activity of alkaline phosphatase is influenced by factors such as initial concentration of substrate (Folley & Kay, 1935; Motzok & Wynne, 1950; Ross, Ely & Archer, 1951; Haab & Smith, 1957; Morton, 1957; Motzok & Branion, 1959), buffers (Aebi & Abelin, 1948; Zittle & Della Monica, 1950), activators (Motzok, 1945; Aebi & Abelin, 1948; Sadasivan, 1952; Morton, 1957; Spencer, 1959; Motzok & Branion, 1959), source of enzyme (Zittle & Della Monica, 1950; Motzok, 1950; Morton, 1955) and type of substrate (Delory & King, 1943; Walker & King, 1950; Motzok, 1959; Moss, 1960). In addition it was shown by Motzok & Branion (1959) that marked differences, and in some cases unexpected similarities, occurred in the pH optima of the alkaline phosphatases of various tissues of different species of animals and birds. Specifically, the data on the phosphatase of intestinal mucosa of mature fowl showed that with 0·05 M-glycerol 2-phosphate maximum activity was obtained at approximately pH 9·7. This is considerably higher than the optimum at pH 9·2 observed previously for the intestinal enzyme of chicks aged 3 weeks, under identical conditions *in vitro* with respect to concentration of substrate, activator (magnesium chloride) and buffer (Motzok, 1950).

Consideration is given in the present paper to the possibility that the optimum pH for the phosphatase of intestinal mucosa, and perhaps of other sources, varies with the age of mammals and birds.

## METHODS AND MATERIALS

Methods for the preparation of tissue phosphatases and for the determination of phosphatase activity have been described previously (Motzok, 1950, 1959). The duodenum of a 1-day-old rat is very small and no attempt was made to remove the mucosa. After washing with 0·9% NaCl soln. and water, the duodena from 25 rats were macerated in a glass Cenco–Potter tissue homogenizer and finally diluted with water to give a concentration of 0·3 g. of tissue (dry wt.)/l. of suspension. Because the liver of rabbits contained appreciable amounts of inorganic phosphorus, disodium phenyl phosphate was used as the substrate and the liberated phenol was determined as a measure of phosphatase activity (Motzok, 1959). In the remainder of the study disodium glycerol 2-phosphate was employed. The activities were measured in the presence of optimum amounts of $MgCl_2$ in the reaction mixtures, i.e. 5 mM for the phosphatases of liver, kidney and bone, and 0·01 M for the enzyme of intestinal mucosa. The reaction period was 20 min. The strains and breeds used in this study were Columbian Rock, White Rock, Barred Plymouth Rock, Australorp and New Hampshire fowl, giant white and black rabbits and Sprague–Dawley and black-hooded rats. The birds and mammals were fed on various experimental and commercial diets.

* Part 2: Motzok & Branion (1959).

## EXPERIMENTAL AND RESULTS

### *Fowl*

*Intestinal mucosa.* In a preliminary study it was observed (Fig. 1*A*) that the phosphatase of intestinal mucosa of Columbian Rock male chicks aged 2 weeks exhibited optimum activity with each concentration of glycerol 2-phosphate at lower pH values than did the enzyme from the intestinal mucosa of hens aged 2 years of the same breed. In the subsequent investigation Columbia male birds were used. The intestinal mucosa from 30 1-day-old chicks was used to establish the initial pH optima. Sixty additional chicks from the same hatch were fed on a commercial chick diet and the enzyme preparations were obtained from groups of 20 birds each, selected at random, at intervals of 6, 8 and 24 weeks. For the last age group 10 cocks, 14 months old, were obtained from the breeder flock maintained at this College. Fig. 1*B* shows that the lowest pH optima were observed with the intestinal enzyme of 1-day-old chicks. The change was to higher pH values for each succeeding age group, and the highest pH optima for the various concentrations of substrate were obtained with the enzyme from the oldest fowls. Fig. 1*C* shows that there were no appreciable differences in pH optima of intestinal phosphatase between sexes and among several breeds of day-old chicks (Columbian Rock, White Rock, Barred Plymouth Rock and New Hampshire) hatched in different seasons of the year.

Consideration was given to the possibility that in preparations of the enzyme such as those used in

these studies, some of the constituents of intestinal mucosa from fowl of different age may influence the characteristics of the enzyme governing the pH required for optimum activity *in vitro*. Appropriate dilutions of intestinal mucosa of additional lots of chicks (2 weeks old) and hens (2 years old) were made to give equal phosphatase activity, i.e. 0·29 and 1 g. of mucosa (dry wt.) respectively per litre. Equal portions of these preparations were mixed and the pH–activity curves with 0·05 M-glycerol 2-phosphate for the two preparations and the mixture were determined 24 hr. and 12 days after storage at 2·5°. Fig. 2 shows that the mixture of the two enzyme preparations exhibited maximum activity at a pH midway between the pH optima for the enzymes of chicks and of mature fowl 24 hr. after the preparations were made. Quantitatively, the maximum activity of the mixture was approximately equal to the activity at the point of intersection of the activity curves of the two preparations determined separately. There were no appreciable differences in the pH–activity curves and the quantitative reaction velocities 12 days after the mixtures were prepared.

In another experiment, the enzyme activity of portions of the two preparations was destroyed by heating at 80° for 30 min. The optimum pH of 9·35 for the enzyme of intestinal mucosa of chicks, with 0·05 M-glycerol 2-phosphate as the substrate, was not affected by the addition of inactivated intestinal mucosa from hens. Similarly, the optimum pH of 9·6 for the enzyme of mature fowl with 0·05 M-substrate was not altered by the addition of inactivated mucosa of chicks.

Although the hens in these studies were fed the same basal diet, the ones used in the preliminary study (Fig. 1*A*) were fed *ad lib.* with a soluble limestone grit, whereas those in the subsequent investigation (Fig. 2) had access to an insoluble granite grit. In view of the difference in pH optima of intestinal phosphatase between the two preparations (i.e. pH 9·8 and 9·6, respectively, with 0·05 M-substrate), an additional experiment was made in which one group of hens was fed *ad lib.* with crushed oyster shell and another group with insoluble granite grit for a period of 4 months. The intestinal phosphatase of the hens fed with oyster shell had optimum activity at pH 9·84 with 0·08 M-glycerol 2-phosphate, and the enzyme of the hens fed granite grit had optimum activity at pH 9·64 with the same concentration of substrate.

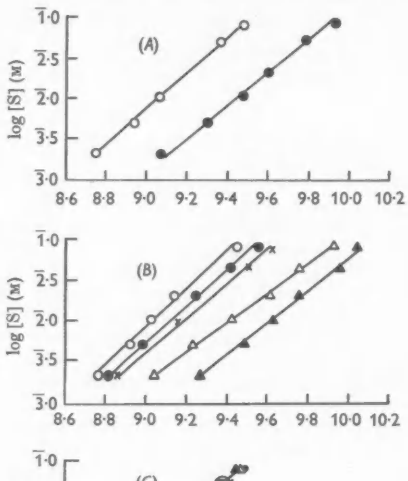


Fig. 1. Relationship between initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the optimum pH for alkaline-phosphatase activity of intestinal mucosa of fowl. (*A*): ○, 2-week-old males; ●, 2-year-old females. (*B*): Males ○, 1 day old; ●, 6 weeks old; ×, 8 weeks old; △, 24 weeks old; ▲, 14 months old. (*C*) 1-day-old chicks: △, New Hampshire males ▲, Columbian Rock females; ●, Barred Plymouth Rock females; ×, Barred Plymouth Rock males; □, White Rock females; ○, White Rock males. Activities were measured in the presence of 0·01 M-$MgCl_2$.

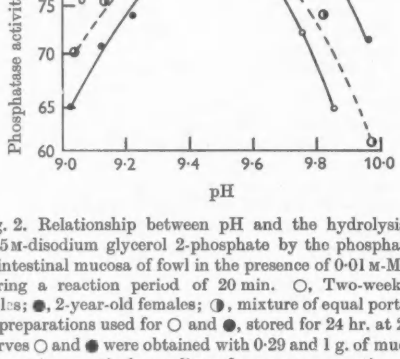


Fig. 2. Relationship between pH and the hydrolysis of 0·05 M-disodium glycerol 2-phosphate by the phosphatase of intestinal mucosa of fowl in the presence of 0·01 M-$MgCl_2$ during a reaction period of 20 min. ○, Two-week-old males; ●, 2-year-old females; ◑, mixture of equal portions of preparations used for ○ and ●, stored for 24 hr. at 2·5°. Curves ○ and ● were obtained with 0·29 and 1 g. of mucosa (dry wt.) respectively per litre of enzyme preparation.

Table 1. pH *optima of alkaline phosphatases of bone, liver and kidney of fowl of various ages*

Activities were measured with disodium glycerol 2-phosphate in the presence of 5 mM-$MgCl_2$ during a reaction period of 20 min. Figures in parentheses are the number of birds used for each enzyme preparation.

| Source of enzyme | Breed of fowl | Sex | Age | No. of enzyme preparations | Optimum pH | |
|---|---|---|---|---|---|---|
| | | | | | 0·08 M [S] | 0·01 M [S] |
| Bone | White Rock | F | 1 day | 1 (25) | 10·04 | 9·54 |
| | Barred Plymouth Rock | M | 5 weeks | 3 (60) | 10·00 | — |
| | Columbian Rock | F | 8 weeks | 10 (20) | — | 9·52–9·56 |
| | White Rock | F | 1 year | 1 (20) | 10·02 | 9·52 |
| Liver | Columbian Rock | F | 2 days | 1 (30) | 10·02 | 9·52 |
| | Columbian Rock | M | 2 days | 1 (30) | 10·02 | 9·52 |
| | Barred Plymouth Rock | M | 5 weeks | 2 (60) | 10·02 | — |
| | Australorp | F | 12 weeks | 3 (20) | 10·02–10·04 | — |
| | Australorp | F | 18 weeks | 2 (20) | 9·98–10·02 | — |
| | White Rock | F | 1 year | 1 (20) | 9·98 | 9·50 |
| Kidney | Columbian Rock | F | 2 days | 1 (30) | 10·04 | 9·52 |
| | Columbian Rock | M | 2 days | 1 (30) | 10·02 | 9·52 |
| | Australorp | F | 12 weeks | 2 (20) | 10·0–10·02 | — |
| | Australorp | F | 18 weeks | 2 (20) | 10·02–10·04 | — |
| | White Rock | F | 1 year | 1 (20) | 10·0 | 9·54 |

*Bone, liver and kidney.* There were no marked differences in pH optima required for the alkaline phosphatases of bone, liver and kidney of fowl with glycerol 2-phosphate as the substrate in the presence of optimum amounts of $Mg^{2+}$ ions in the reaction mixture (Table 1). For example, the enzymes of bone, liver and kidney of several breeds of fowl (White Rock, Columbian Rock, Barred Plymouth Rock and Australorp), varying in age from 1 day to 1 year and fed on different experimental and commercial diets, exhibited optimum activity at pH 9·98–10·04 with 0·08 M-substrate. There were also no appreciable differences between sexes.

### *Rabbits*

*Intestinal mucosa.* The optimum pH for the alkaline phosphatase of intestinal mucosa of rabbits with each concentration of glycerol 2-phosphate was quite high when the animals were 2 days old and the change in pH optima was to lower values for the older animals (Fig. 3*A*).

*Bone, liver and kidney.* There was no appreciable change due to age (2-day-old and mature rabbits) in the pH optima for the phosphatases of bone (Fig. 3*B*) and kidney (Fig. 3*C*) with glycerol 2-phosphate and for the enzyme of liver (Fig. 3*D*) with phenyl phosphate as the substrate.

### *Rats*

There were no differences in pH optima with each concentration of substrate among the phosphatase preparations of intestinal mucosa from rats aged 6 weeks, 6 and 12 months (Fig. 4). The pH optima for the phosphatase of the whole duodenum of 1-day-old rats were higher than those of the mucosal enzyme of the older rats.

## DISCUSSION

The optimum pH for the phosphatase of intestinal mucosa of fowl changed to more alkaline conditions for each succeeding age group. The process is a gradual one and the higher pH optimum with each concentration of substrate was attained at about 6–12 months of age. There were no appreciable differences in pH optima of intestinal phosphatase between sexes and among several breeds of day-old chicks. Some differences, however, have been observed among groups of older fowl of the same age but fed different commercial and experimental diets. The suggestion that variations in the mineral portion of the diet causes at least a part of these differences is based on the observation that the optimum pH of intestinal phosphatase was lower in hens given insoluble granite grit than in hens given limestone or crushed oyster shell.

The difference in the optimum pH for the intestinal enzyme of chicks and mature fowl cannot be attributed to any constituent of the enzyme preparations which is not destroyed by heating at 80°. Further, the pH–activity curve and the quantitative reaction velocities of a mixture of the preparations appear to be of the type one would expect to obtain with a combination of two enzymes possessing different pH optima.

In rabbits the pH optima for the phosphatase of intestinal mucosa also changed with each succeeding age group, but, in contrast with fowl, the shift was to lower values. The pH optima for the intestinal enzyme of rabbits at 2 days of age were the highest observed in these studies and were comparable to the pH optima for the phosphatases of rabbit liver and kidney. Indeed the pH optima

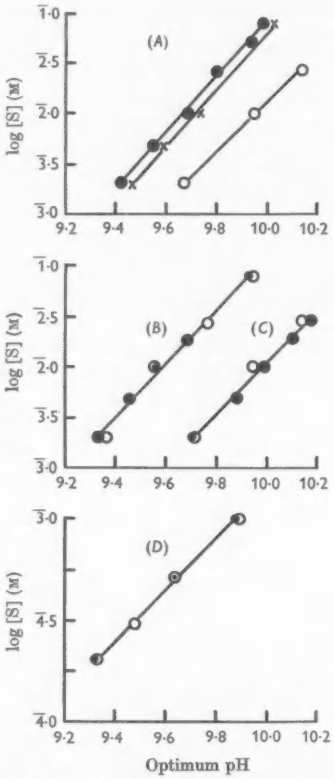


Fig. 3. Relationship between initial concentration of substrate [S], expressed logarithmically, and the optimum pH for activity of alkaline phosphatases of rabbits. (*A*), Intestinal mucosa; (*B*), bone; (*C*) kidney (disodium glycerol 2-phosphate as substrate); (*D*), liver (disodium phenyl phosphate as substrate). Age of rabbits: ○, 2 days; ×, 7 weeks; ●, 1 year. Activities were measured in the presence of optimum concentrations of $MgCl_2$ in the reaction mixtures: 5 mM for the phosphatases of bone, kidney and liver; 0·01 M for the phosphatase of intestinal mucosa.

for high concentrations of substrate could not be determined because these were beyond the effective range of the buffer system used. The major change in pH optima appeared to take place in the first two months of life.

In contrast with the definite change in pH optima of intestinal phosphatases of fowl and rabbits, the pH optima for the enzymes of bone, liver and kidney were quite constant in these species regardless of their age. There appears to be no information in the literature which would account for the change in pH optima of phos-

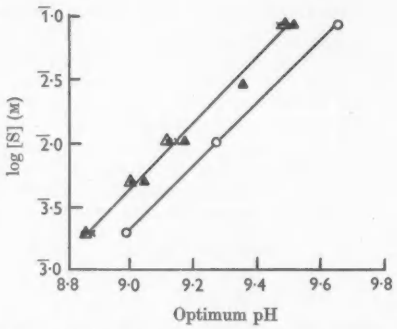


Fig. 4. Relationship between initial concentration of disodium glycerol 2-phosphate, expressed logarithmically, and the pH for optimum activity of intestinal alkaline phosphatase of rats. Age of animals: ○, 1 day (whole duodenum); ●, 6 weeks; ×, 3 months; △, 6 months; ▲, 1 year (intestinal mucosa). Activities were measured in the presence of 0·01 M-$MgCl_2$.

phatase in intestinal mucosa and not in other tissues. A possible explanation may be that the intestinal mucosa becomes exposed to a slurry of food containing a variety of dietary substances in concentrations which are different from those supplied by the egg of fowl during hatching or from maternal source in the uterus in mammals. If the concentration of specific nutrient in the 'environmental media' in intestinal tissue has an effect on the properties of mucosal phosphatase during its biogenesis, a gradient in the substance provided by the diet would result in the production of new phosphatase with characteristics different from those of the enzyme originally present in the mucosa.

There was no change in pH optima for phosphatase of intestinal mucosa of rats as their age increased from 6 weeks to 1 year. Although it may be assumed that most of the phosphatase of the preparation of whole duodenum (1-day-old rats) was of mucosal origin, a comparison of optimum pH for the enzyme of such a preparation with those of preparations of mucosa of older animals (Fig. 4) may not be justified.

## SUMMARY

1. The pH optima for the phosphatase of intestinal mucosa of fowl (for the respective concentrations of glycerol 2-phosphate) rose with increasing age of the birds. There were no differences between sexes and among several breeds of 1-day-old chicks.

2. The optimum pH for the intestinal phosphatase of chicks was not affected by the addition

of inactivated (heated at 80° for 30 min.) intestinal mucosa of hens, and the optimum pH for the enzyme of mature fowl was not altered by inactivated mucosa of chicks.

3. A mixture of the enzyme preparations of chicks and mature fowl exhibited a pH–activity curve and quantitative reaction velocities which would be expected from a combination of two enzymes possessing different pH optima.

4. In rabbits the shift in pH optima for intestinal phosphatase was to lower values with increasing age, the major change taking place in the first 2 months of life.

5. There was no change in pH optima of the phosphatase of intestinal mucosa of rats as their age increased from 6 weeks to 1 year. The pH optima for the phosphatase of a preparation of the whole duodenum of 1-day-old rats were higher than those of the mucosal enzyme of older rats.

6. The pH optima for the phosphatases of bone, liver and kidney of fowl and rabbits appeared to be constant regardless of their age.

The authors wish to express their indebtedness to Miss G. Ritcey for invaluable technical assistance.

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# Protein Synthesis in Mitochondria

## REQUIREMENTS FOR THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO MITOCHONDRIAL PROTEIN

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Keller (1951) observed that after the injection of radioactive leucine into the rat the most radioactive protein in the subcellular components of the liver was in the microsome fraction. After this Siekevitz (1952) found that the most efficient method *in vitro* for obtaining incorporation of radioactive alanine into protein was to incubate a mixture of microsomes (precipitated at pH 5) and mitochondria in a medium that allowed the mitochondria to carry out oxidative phosphorylation. He suggested that the main function of the mitochondria was to provide energy for the microsomal system and this view was strengthened by the observation of Zamecnik & Keller (1954) that the mitochondria could be replaced by cell sap and an adenosine triphosphate-generating system. Because of low rates of labelling of the mitochondria observed in this early work, the high microsomal contamination in the mitochondrial preparations used, and the high proportion of radioactivity lost from the protein by treatment with ninhydrin (Rutman, Cantarow & Paschkis, 1954) or mercaptoethanol (Peterson & Greenberg, 1952), the mitochondria were ignored for several years as an active site for protein synthesis, and their function was regarded primarily as an energy donor for the microsomal system (see, for example, Brachet, 1958).

More recently, however, evidence has appeared that mitochondria are capable of independent protein synthesis. McLean, Cohn, Brandt & Simpson (1958) found that, in contrast with rat liver, skeletal muscle showed equivalent rates of incorporation of radioactive leucine into mitochondrial and microsomal protein *in vivo*. Equivalent rates of labelling of mitochondria and microsomes *in vivo* have also been observed in ascites cells (E. M. Martin, personal communication). The incorporation of radioactive amino acids into the

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protein of isolated plant mitochondria has been reported (Sisakyan & Filippovich, 1957), of mitochondria from *Tetrahymena pyriformis* (Mager, 1960), of mitochondria from *Paracentrotus lividus* (Giudice, 1960), of liver mitochondria (McLean *et al.* 1958; Greengard, 1959; Greengard & Campbell, 1959; Rendi, 1959*a*, *b*; Korner, 1959) and of skeletal muscle mitochondria (McLean *et al.* 1958). Fraser & Gutfreund (1958) observed similar rates of labelling *in vitro* with microsomal and mitochondrial fractions isolated from mammary tissue. The most striking observations, however, have been the demonstration of the incorporation of radioactive valine into cytochrome *c* by isolated rat-liver mitochondria (Bates, Craddock & Simpson, 1958, 1960) and of the net synthesis of cytochrome *c* over a period of 12 hr. by a preparation of calf-heart sarcosomes (Bates & Simpson, 1959).

Rat-liver mitochondria have been chosen for this study because of the information available as to their isolation and biochemical properties (Schneider, 1959), and in this paper their requirements for incorporation of radioactive amino acids into proteins have been investigated. Some of our preliminary observations have been published elsewhere (Reis, Coote & Work, 1959).

## METHODS

### *Cell fractionation*

The cell fractions were isolated between 0° and 4° in 0·3 M-sucrose–2 mM-ethylenediaminetetra-acetate (EDTA) (disodium salt)–0·03 M-nicotinamide adjusted to pH 7·1 with KOH. Young rats, weighing between 100 and 120 g., were starved for 15 hr. and killed by a blow on the head followed by decapitation. The livers were rapidly removed, washed in 0·15 M-NaCl followed by the sucrose–EDTA–nicotinamide medium and then forced through a tissue mincer containing a stainless-steel screen with holes 1 mm. in diameter. The mince was suspended in 3 vol. of medium and homogenized in a Potter–Elvehjem homogenizer with a $1\frac{1}{4}$ in. diam. polytetrafluoroethylene (Teflon) pestle (clearance between pestle and mortar 0·02–0·03 in.) with 8–10 slow strokes, the pestle rotating between 1500 and 2000 rev./min. The homogenate was filtered through plain surgical gauze to remove pieces of fibre and clumps of unbroken cells. Nuclei, whole cells and red blood cells were removed by sedimenting at 700*g* for 15 min.

Mitochondria were prepared from the resulting supernatant by five successive sedimentations at 8000, 7000, 6000, 8000 and 8000*g*, each for 10 min. Before the last sedimentation at 8000*g*, the resuspended mitochondria were centrifuged at 700*g* for 6 min. to remove any residual nuclei and red blood cells. The mitochondria were resuspended between sedimentations as gently as possible by hand in a Potter–Elvehjem homogenizer, with 5–10 slow strokes of the pestle.

Radioactive microsomal fraction was prepared from the liver of a rat injected 30 min. previously with 10 $\mu$c of algal protein hydrolysate. The supernatant, after the mitochondria had been sedimented, was centrifuged at 105 000*g* for 60 min. and the pellet washed free of supernatant fluid by gently swirling fresh medium over it. The resuspended microsomal pellet was then centrifuged at 8000*g* for 10 min. in order to remove any clumped material.

Cell sap was taken as the final supernatant after centrifuging at 105 000*g*; 4·0 ml. of cell sap was derived from 1 g. wet wt. of liver mince.

Acetone-dried powders were prepared by slowly adding a suspension of mitochondria in water to 10 vol. of AnalaR acetone at −15°. The precipitate was washed with cold acetone and dried. The dry powder was homogenized in buffer and the suspension centrifuged at 8000*g* for 10 min. For demonstration of adenosine triphosphate (ATP)–pyrophosphate exchange (Table 2) the buffer used was 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl, pH 7·6, and for separation of soluble and insoluble protein for radioactivity determinations (Fig. 1) 0·1 M-potassium phosphate, pH 7·0, was used.

For measuring bacterial contamination, suitable quantities of the homogenate, mitochondrial fraction or final reaction mixture were diluted with sterile 0·9% NaCl soln. and 0·1 ml. was plated on blood–agar plates. Colonies were counted after 18 hr. incubation at 37°.

### *Incubation conditions for studying amino acid incorporation*

The mitochondria were incubated in 3–6 ml. of the appropriate medium generally at 30° under $O_2$, in 50 ml. conical flasks shaken at 100 cyc./min.; 10–20 mg. of mitochondrial protein was added per 3 ml. of reaction mixture. The reaction was stopped by addition of carrier amino acid, followed by trichloroacetic acid to a final concn. of 5% (w/v), and the protein then isolated for plating by the method of Simkin & Work (1957*a*).

The composition of the reaction mixture was modified as the work developed. However, all experiments were done with a basal medium (*Medium A*: this contained 0·1 M-sucrose, 0·04 M-KCl, 1·3 mM-EDTA, 0·02 M-nicotinamide and 0·01 M-potassium succinate) supplemented with the additions described in the legends to the appropriate figures and tables. In some experiments 0·01 M-potassium α-oxoglutarate was used as an energy source instead of succinate, and this is indicated in the legends to the appropriate tables and figures. Many of the later experiments were done with a more complex medium (*Medium B*: this was basal medium A + 0·016 M-potassium phosphate, 4 mM-adenosine monophosphate, 0·5 mM-diphosphopyridine nucleotide (DPN), 8 mM-$MgSO_4$ and 50 $\mu$g. of synthetic amino acid mixture/ml.). The synthetic amino acid mixture had similar molar proportions to that found in cell sap (alanine 1·0, arginine 0·125, aspartic acid 0·5, asparagine 0·25, cystine 0·5, glutamic acid 1·0, glutamine 0·75, glycine 1·0, histidine 0·25, isoleucine 0·375, leucine 0·375, lysine 0·5, methionine 0·5, phenylalanine 0·125, proline 0·25, serine 0·75, threonine 0·5, tryptophan 0·125, valine 0·5 and tyrosine as a saturated solution) and was passed through the resin Amberlite IRC-50 ($K^+$ form) before use in order to remove heavy metals. The pH of all the incubation media was adjusted to pH 7·0–7·2 with KOH. In some experiments 0·02 M-glucose and (crude) hexokinase (80 $\mu$g./ml.) were added in order to lower the concentration of the extramitochondrial ATP and hence minimize the effect of microsomal contamination. With α-oxoglutarate as an

energy source this did not affect the incorporation, but, for a reason as yet unknown, some inhibition was obtained when succinate was used.

### *Enzyme preparations and assay methods*

pH 5 Enzyme was prepared by the method of Keller & Zamecnik (1956).

Two samples of ribonuclease were used, one from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and the other, a highly purified sample, kindly provided by Dr R. R. Porter.

Glucose 6-phosphate dehydrogenase was obtained from C. F. Boehringer und Söhne GmbH., Mannheim, Germany.

A crude sample of hexokinase was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Determination of P:O ratios was carried out with a sample of twice recrystallized yeast hexokinase kindly provided by Dr R.A. Darrow and Dr S. P. Colowick.

Glucose 6-phosphatase was estimated by incubating the tissue at 30° for 10 min. in the presence of 4 mM-glucose 6-phosphate–0·03 M-sodium succinate, pH 6·6. The liberation of inorganic phosphate was followed.

Succinoxidase was estimated manometrically by the method of Schneider & Potter (1943), except that $AlCl_3$ was omitted from the reaction mixture.

Adenosine triphosphate–pyrophosphate exchange was followed by measuring the incorporation of [$^{32}$P]pyrophosphate into ATP. The incubation conditions were as described by Hoagland (1955) (with an equimolar mixture of 12 amino acids) and ATP was isolated from the reaction mixture by the method of Crane & Lipmann (1953). Percentage exchange was taken as

$$\frac{\text{Total counts/min. in ATP}}{\text{Counts/min. in [}^{32}\text{P]pyrophosphate added}} \times 100.$$

Oxidative phosphorylation was measured by the appearance of glucose 6-phosphate after incubation in the presence of glucose and hexokinase (Slater, 1953). Glucose 6-phosphate was estimated spectrophotometrically with triphosphopyridine nucleotide (TPN) and glucose 6-phosphate dehydrogenase, according to the method of Horecker & Wood (1957). For the measurement of P:O ratios, crystalline hexokinase was used, and $O_2$ uptake was measured manometrically and extrapolated to the time the mitochondria were added, according to the method of Slater & Holton (1953).

### *Chemical estimations*

Acid-soluble material, ribonucleic acid (RNA), lipid and protein were separated essentially according to the method of Schneider (1945).

Inorganic phosphate and total phosphate were determined by the method of Fiske & Subbarow (1925).

Phospholipid was estimated by its total phosphate content and it was assumed that 1 mg. of phospholipid P corresponded to 25 mg. of phospholipid (Siekevitz & Watson, 1956).

Ribonucleic acid was determined by its pentose content with the orcinol reagent (Mejbaum, 1939) as described by Umbreit, Burris & Stauffer (1957). Adenosine monophosphate was used as a standard and it was assumed to give twice the extinction of an equivalent amount of RNA (Dische, 1955). It was also assumed that 1 $\mu$mole of RNA-pentose was equivalent to 31 $\mu$g. of RNA-phosphorus (RNA-P).

Protein was estimated by its N content by using a micro-Kjeldahl procedure.

*α-Amino nitrogen of cell sap and mitochondria.* Cell sap was heated at 100° for 5 min. and the coagulated protein removed by centrifuging. α-Amino N was determined directly on the clear supernatant. Mitochondria were extracted with cold 5% (w/v) trichloroacetic acid and the extract was shaken with ether several times. The aqueous phase was taken for analysis. The Van Slyke technique was used (Van Slyke, 1929; Van Slyke, Dillon, MacFadyen & Hamilton, 1941).

*Isolation of amino acids from cell sap.* The supernatant from boiled cell sap was desalted by loading on the ion-exchange resin Zeo-Karb 225 ($H^+$ form). The amino acids were eluted with aq. N-$NH_3$ soln. and the eluate was evaporated to dryness. Samples were taken for studying the effect on amino acid incorporation (Table 1) and the remainder was analysed by two-dimensional descending paper chromatography with aq. 71% (v/v) phenol, in an atmosphere saturated with $NH_3$ in the first direction and butan-1-ol–acetic acid–water (63:10:27, v/v) in the second. The chromatograms were developed by spraying with 0·1% (w/v) ninhydrin in 98% butanol and the relative amounts of amino acids estimated from the size and colour intensity of the spots.

### *Radioactivity determinations*

*$^{32}$P content of adenosine triphosphate.* This was determined in a liquid counter (Veall, 1948) and suitable precautions were taken to allow for the decay of $^{32}$P in calculation of the results.

*Radioactive protein.* This was counted at infinite thickness as compressed disks, with either 1 sq.cm. polythene or 0·3 sq.cm. polymethyl methacrylate (Perspex) planchets. End-window Geiger–Müller counters were used and standard Perspex sources of radioactivity obtained from The Radiochemical Centre, Amersham, Bucks., were counted in parallel with the protein samples. A 1 sq.cm. standard containing 1000 $\mu$mc/g. gave 950 counts/min. with the apparatus used. In all experiments a zero-time assay was carried out by adding carrier amino acid before adding the mitochondria and then immediately adding trichloroacetic acid. This zero-time value was then subtracted from all the values found for radioactivity of protein. With 0·33 $\mu$c/ml. of algal protein hydrolysate the zero-time value was 10·6 $\mu$mc/g. (average of 24 determinations). With 1·0 $\mu$c/ml. of [$^{14}$C]valine it was 4·2 $\mu$mc/g. (average of six determinations).

*Radioactive chemicals.* [$^{32}$P]Pyrophosphate was prepared by pyrolysis of inorganic [$^{32}$P]phosphate obtained from The Radiochemical Centre, Amersham, Bucks. (Kornberg & Pricer, 1951). Uniformly labelled L-[$^{14}$C]-valine (1 mc/17·9 mg.) and [$^{14}$C]algal protein (1 mc/4·5 mg.) were also obtained from The Radiochemical Centre. The algal protein was hydrolysed and the amino acids obtained were purified by the method of Simkin (1958).

Where possible all reagents were recrystallized before use. All solutions were made up in de-ionized distilled water.

## RESULTS

*Development of incubation medium for studying amino acid incorporation into mitochondrial protein*

*Effect of cell sap.* If mitochondria isolated in 0·25 M-sucrose (Schneider, 1948) were incubated in the presence of radioactive amino acids in relatively simple media (e.g. tris–$Mg^{2+}$ ions–succinate mixtures) little or no incorporation was observed. The incorporation was greater if EDTA (Cleland & Slater, 1953) and nicotinamide [to prevent DPN breakdown (Kaplan, 1955)] were added to the isolation medium and under these conditions cell sap was necessary for incorporation. However, if the whole cell sap was heated at 100° for 5 min. and the coagulated protein centrifuged down, the clear supernatant was as effective as the untreated cell sap in maintaining incorporation (Table 1). Treatment of whole cell sap with ribonuclease was not inhibitory, nor was treatment with ribonuclease followed by boiling. These results suggested that the effect of cell sap was due to substances of low molecular weight. Examination revealed the presence of considerable quantities of adenosine monophosphate and DPN and when these were added improvement in amino acid incorporation and in phosphorylation were observed. However, the main effect of the cell sap seemed to be due to the amino acids present. Van Slyke analysis showed 81 $\mu$g. of $\alpha$-amino nitrogen (approx. 0·7 mg. of amino acid/ml.) and isolation on Zeo-Karb 225 ($H^+$ form) followed by chromatography showed a full complement of amino acids in the approximate molar proportions given in the Methods section (see 'Incubation conditions for studying amino acid incorporation'). These isolated amino acids could effectively replace the boiled cell sap in maintaining incorporation (Table 1).

Table 1. *Effect of various treatments of cell sap on incorporation of radioactive amino acids into mitochondrial proteins*

Mitochondria were incubated at 37° under the following conditions. Expt. 1: medium A plus 0·01 M-potassium phosphate, 0·07 M-tris–HCl, 0·012 M-$MgSO_4$, 6 mM-adenosine monophosphate, 0·5 mM-DPN, 0·15 ml. of cell sap and 1·25 $\mu$c of algal protein hydrolysate/ml.; incubation in air. Expt. 2: medium A plus 0·01 M-potassium phosphate, 0·07 M-tris–HCl, 5 mM-$MgSO_4$, 0·15 ml. of cell sap and 1·25 $\mu$c of algal protein hydrolysate/ml. Expt. 3: as in Expt. 2 except with 1·5 $\mu$c of [$^{14}$C]valine/ml. Expt. 4: medium B plus glucose and crude hexokinase, and $\alpha$-oxoglutarate as energy source; 0·3 ml. of cell sap and 1·0 $\mu$c of algal protein hydrolysate/ml.

| Expt. no. | Time of incubation (min.) | Radioactivity in protein ($\mu$mc/g.) Whole cell sap | Boiled cell sap | Amino acids from boiled cell sap |
|---|---|---|---|---|
| 1 | 30 | 84, 86, 88 | 87, 89 | — |
| | 60 | 112 | 110 | — |
| 2 | 15 | 73 | 96 | — |
| | 30 | 92 | 97 | — |
| | 60 | 100 | 109 | — |
| 3 | 30 | — | 21·5 | 19·0 |
| 4 | 60 | — | 60 | 59 |

*Role of amino acid-activating enzymes.* Since the effect of cell sap is not destroyed by boiling, it is unlikely that added activating enzymes can stimulate mitochondrial incorporation, and it has not been possible to observe any effect with added pH 5 enzyme. It is possible, however, that mitochondrial amino acid-activating enzymes are involved. It was difficult to demonstrate any ATP–pyrophosphate exchange that is dependent on amino acids if intact or disrupted mitochondria were used, because of the high level of non-specific exchange. However, some amino acid dependence was observed in extracts of acetone-dried powders, which showed much less non-specific exchange, but the enzyme activity was smaller than with pH 5 enzyme (Table 2).

*Amino acid concentration in mitochondria and medium.* Van Slyke analysis and chromatography showed the presence of a full complement of free

Table 2. *Amino acid-dependent exchange between adenosine triphosphate and pyrophosphate in various preparations*

Mitochondria were disrupted with ultrasonic vibrations (25 kcyc./sec. for 2 min. at 0°). Acetone-dried powder was extracted with 0·1 M-tris–HCl, pH 7·6.

| Prep. | Wt. of liver used to obtain preparation (mg.) | Exchange (%) No amino acids | Plus amino acids | Increment due to amino acids |
|---|---|---|---|---|
| Intact mitochondria | 310 | 20·4 | 20·9 | +0·5 |
| Disrupted mitochondria | 280 | 23·8 | 24·7 | +0·9 |
| Extract of acetone-dried powder | 350 | 4·8 | 6·4 | +1·6 |
| | 920 | 10·7 | 14·0 | +3·3 |
| pH 5 enzyme | 120 | 19·0 | 37·5 | +18·5 |

amino acids in the mitochondria (3·3 $\mu$g. of $\alpha$-amino nitrogen or approx. 28 $\mu$g. of amino acid/mg. of protein). Since 10–20 mg. of mitochondrial protein was added to the incubation flasks, it would be expected that milligram quantities of radioactive amino acid would be needed to saturate the mitochondrial pool. In confirmation of this it was observed that maximum labelling of the mitochondrial protein was not obtained until 2 mg. of amino acid/ml. was added, and even then only in insoluble and not in soluble proteins (Fig. 1).

Unfortunately the amount of incorporation obtained with the mitochondria does not permit the routine use of such high concentrations of amino acid, since exorbitant amounts of radioactivity would have to be added to obtain measurable counts. We have found that adding 150 $\mu$g. of synthetic amino acid mixture and 1 $\mu$c of [$^{14}$C]-algal hydrolysate/3 ml. of reaction mixture gives a convenient final radioactivity in the protein of 50–100 counts/min. after incubation for 1 hr., but these amounts are entirely empirical and the system is far removed from saturation with respect to amino acid concentration.

If it is assumed that the intramitochondrial amino acids equilibrate freely with those in the medium, the average specific radioactivity of the amino acids in the system can be calculated from the amounts of mitochondria, [$^{14}$C]amino acid and [$^{12}$C]amino acid added, assuming the presence of 28 $\mu$g. of amino acid/mg. of protein. If the effect of adding low concentrations of radioactive amino acid (0–200 $\mu$g./ml.) is studied in this way, it is observed that the final radioactivity of the protein is directly proportional to the (calculated) specific radioactivity of the amino acids (Fig. 2). Over this range of amino acid concentration, therefore, alterations in the amount of amino acid added do not affect the net rate of protein synthesis, but merely alter the extent of labelling of the pool.

*Energy source.* In all the experiments, we have relied upon oxidative phosphorylation by the mitochondria to provide energy for the incorporation process. The dependence of the system on oxidative phosphorylation is shown by greatly reduced incorporation on incubation under anaerobic conditions, in the presence of 0·2 mM-2:4-dinitrophenol, 1 mM-potassium cyanide, or in the absence of inorganic phosphate or an oxidizable substrate (succinate or $\alpha$-oxoglutarate) (Table 3). The optimum concentrations of inorganic phosphate, $\alpha$-oxoglutarate and succinate are shown in Fig. 3. On several occasions it was possible to observe simultaneous failure in incorporation and phosphorylation (see Fig. 1 in Reis *et al.* 1959).

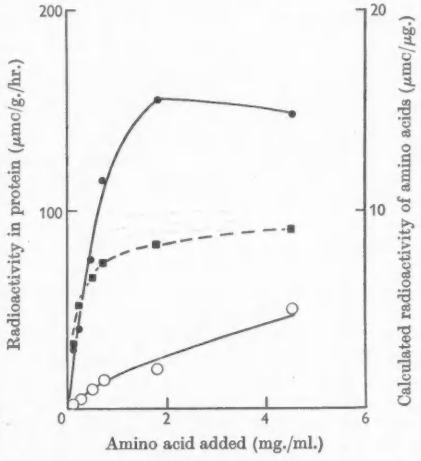


Fig. 1. Effect of amino acid concentration on radioactivity of mitochondrial protein. ●, Residual protein; ○, extracted protein; ■, calculated radioactivity of pool. Mitochondria were incubated for 1 hr. in medium B in the presence of glucose and crude hexokinase and $\alpha$-oxoglutarate as energy source. Mixtures of algal protein hydrolysate and synthetic amino acid mixture were added in constant proportion (9·1 $\mu$mc/$\mu$g.) in the amounts shown. Radioactivity of pool was calculated from amounts of amino acid added and present in mitochondria and assuming free equilibration between mitochondria and medium. Acetone-dried powders were prepared at end of incubation and extracted with 0·1 M-potassium phosphate buffer, pH 7·0.

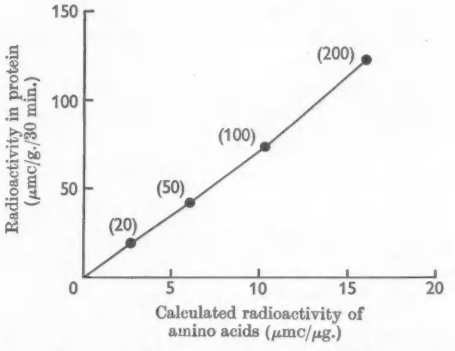


Fig. 2. Relationship between labelling of mitochondrial protein and (calculated) specific radioactivity of pool. Mitochondria were incubated for 30 min. in medium A plus 0·01 M-potassium phosphate, 0·02 M-tris–HCl, 6 mM-$MgSO_4$ and mixtures of algal protein hydrolysate and synthetic amino acid mixture in constant proportion (50 $\mu$mc/$\mu$g.) added in amounts ($\mu$g./ml.) shown in parentheses. Specific radioactivity of pool were calculated as in Fig. 1.

*Nucleotides and* $Mg^{2+}$ *ions.* Addition of DPN results in some improvement in incorporation (Table 3) but there appears to be no absolute requirement, probably because of the presence of endogenous nucleotide. The effect of addition of adenosine monophosphate and $MgSO_4$ is more impressive, however. The optimum concentrations of $MgSO_4$ and adenosine monophosphate are shown in Fig. 4 and a ratio of approx. 2 moles of $MgSO_4$ to 1 mole of adenosine monophosphate was most satisfactory.

*Mitochondrial concentration.* If the amount of radioactive amino acid added is kept constant the final radioactivity of the protein falls as the mito-

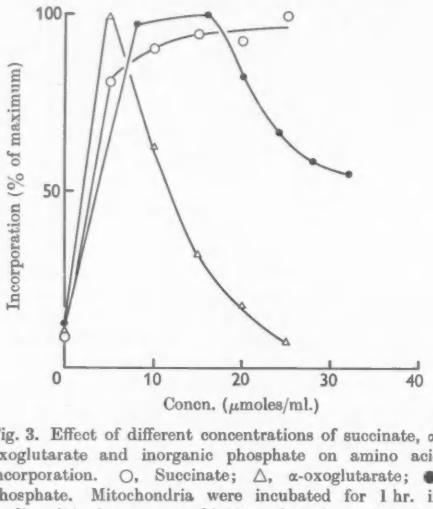


Fig. 3. Effect of different concentrations of succinate, α-oxoglutarate and inorganic phosphate on amino acid incorporation. ○, Succinate; △, α-oxoglutarate; ●, phosphate. Mitochondria were incubated for 1 hr. in medium B in the presence of 0·33 μc of algal protein hydrolysate/ml. Maximum incorporation with succinate was 115 μmc/g./hr., α-oxoglutarate 104 μmc/g./hr. and phosphate 121 μmc/g./hr.

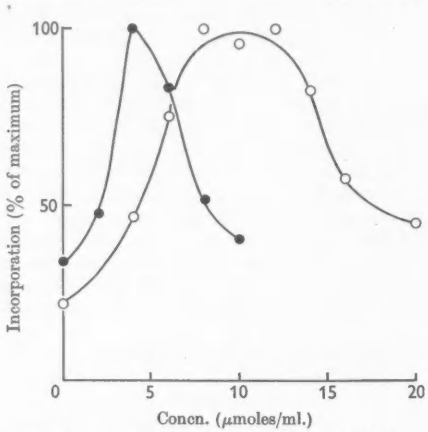


Fig. 4. Effect of different concentrations of $MgSO_4$ and adenosine monophosphate on amino acid incorporation. ○, $MgSO_4$; ●, adenosine monophosphate. Mitochondria were incubated for 1 hr. in medium B in the presence of 0·33 μc of algal protein hydrolysate/ml. Maximum incorporation with $MgSO_4$ was 98 μmc/g./hr., with adenosine monophosphate 48 μmc/g./hr.

Table 3. *Effect of inhibitors and of omission of components of incubation medium on incorporation of amino acids*

Mitochondria were incubated in medium B plus 0·33 μc of algal protein hydrolysate/ml. Results are expressed as percentage of radioactivity in protein after incubation for 1 hr. in the complete system.

| | Radioactivity in protein | |
|---|---|---|
| | 1 hr. incubation | 2 hr. incubation |
| Complete system | 100·0 | 127·0 |
| − DPN | 72·0 | 97·0 |
| − AMP | 59·8 | 77·0 |
| − $MgSO_4$ | 16·2 | 13·4 |
| − α-Oxoglutarate* | 16·4 | 14·8 |
| − Succinate | 4·4 | 19·0 |
| − Phosphate | 11·3 | 33·3 |
| Sucrose medium only | 15·2 | 17·3 |
| + 0·2 mM-2:4-Dinitrophenol | 11·0 | — |
| + 1 mM-KCN | 8·3 | 11·8 |
| Incubation under $N_2$ | 10·8 | — |

* Complete system contained α-oxoglutarate plus glucose and crude hexokinase. Succinate was used as energy source in all other experiments.

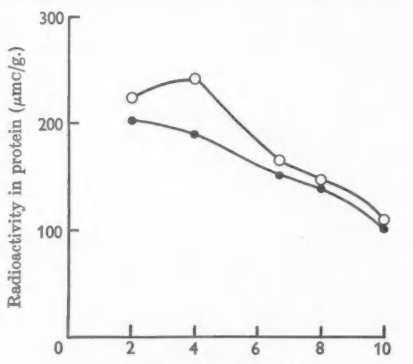


Fig. 5. Effect of concentration of mitochondrial suspension on incorporation of amino acids into mitochondrial protein. ●, Incubation for 1 hr.; ○, incubation for 2 hr. Mitochondria were incubated in medium B plus 0·33 μc of algal protein hydrolysate/ml.

chondrial concentration is raised (Fig. 5). This is to be expected if the mitochondria contain a significant pool of endogenous amino acid. However, it also appears from Fig. 5 that the duration of the incorporation (as judged by the difference between incubation for 1 and 2 hr.) is affected by mitochondrial concentration, the system being most active at a concentration of 4 mg. of protein/ml.

### *Behaviour of mitochondria in final medium used (medium B)*

*Oxygen uptake and phosphorylation.* Medium B, which was developed to obtain the best amino acid incorporation, bears a strong resemblance to media generally used for the study of oxidative phosphorylation. With this medium, lower oxygen uptakes were observed with succinate as a substrate than in the succinoxidase assay of Schneider & Potter (1943) [280 $\mu$l. of $O_2$/hr./mg. of protein N compared with 2100 $\mu$l. of $O_2$/hr./mg. of protein N], indicating that the mitochondria do not exhibit their full oxidative capacity. The oxygen uptake is very low if 0·01 M-glucose is used instead of the succinate (19 $\mu$l. of $O_2$/hr./mg. of protein N). The inability of the preparations to oxidize glucose has been of use in studying the problem of bacterial contamination (Fig. 9). Under the conditions normally used for studying amino acid incorporation, P:O ratios of 0·9–1·1 were generally obtained if crystalline hexokinase was used in the assay. If the mitochondria were incubated for short periods of time (10–20 min.) in more dilute suspension than was normally used, more satisfactory P:O ratios could be obtained (1·5–1·7).

*Amino acid incorporation.* Considerable variation was observed in the initial rate of incorporation and in the shape of the progress curve. In general, incorporation is linear for about 1 hr. and then falls off steadily, finally ceasing after incubation for 1½–2 hr. at 30°. In some experiments, however, the incorporation ceased abruptly after 1 hr., whereas in others it continued for 2½ hr. With medium B and succinate as an energy source the rate of labelling of mitochondrial protein was 83·0 ± 7·9 $\mu$mc/g./hr. (eight determinations), in the presence of 0·33 $\mu$c of algal protein hydrolysate/ml. Addition of glucose and crude hexokinase lowered this (57·5 ± 8·5 $\mu$mc/g./hr.) (13 determinations). With medium B and 1·0 $\mu$c of [$^{14}$C]valine/ml. as a label the rate of labelling was 62·7 ± 8·0 $\mu$mc/g./hr. (6 determinations). In the absence of direct measurements of the specific radioactivity of the intramitochondrial pool of amino acids, the observed radioactivity in the protein cannot be expressed in terms of weight of amino acid incorporated. However, by using the figure of 28 $\mu$g. of amino acid/mg. of protein for the size of the pool, from the amount of amino acid added and the mitochondrial concentration, and assuming free equilibration of amino acids across the mitochondrial membrane, it can be calculated that the mean rate of incorporation with algal protein hydrolysate as a label (83 $\mu$mc/g./hr.) is equivalent to 45 $\mu$g. of amino acid/g. of protein/hr., i.e. one amino acid residue in 22 000.

Improvement in this figure depends on increasing the time for which incorporation can continue. Preliminary experiments suggested that alteration in the composition of the medium was a more likely cause for failure than inactivation of the mitochondria. Preincubated mitochondria were almost as active as fresh mitochondria if they were resuspended in fresh medium. However, fresh mitochondria were less active in the medium obtained from preincubated mitochondria than in fresh medium. It was then found that if the medium was changed every hour, there was considerable improvement in the stability of the system. Table 4 shows that both phosphorylation and amino acid incorporation continue for 3 hr. under these conditions.

*Site of labelling of mitochondria.* Fig. 1 shows that the proteins that are extracted by dilute phosphate buffer from acetone-dried powders of mitochondria are less radioactive than the residual protein. Also, during incubation a certain amount of protein is lost from the mitochondria into the medium and this protein has a lower radioactivity than that retained within the mitochondria.

Disruption of mitochondria with the non-ionic detergent Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) confirmed that the insoluble proteins (sedimented at 105 000$g$ for 60 min.) are more radioactive than the soluble

Table 4. *Stability of amino acid incorporation and phosphorylation in mitochondria incubated in medium B*

Results are expressed as a percentage of the value after incubation for 1 hr. (45·0 $\mu$mc/g. and 5·82 $\mu$moles of glucose 6-phosphate/ml. of reaction mixture). Mitochondria were incubated in medium B in the presence of 0·33 $\mu$c of algal protein hydrolysate/ml., 0·02 M-glucose and 80 $\mu$g. of crude hexokinase/ml. Medium was changed by centrifuging the suspension at 8000$g$ for 10 min. after incubation for 1 and 2 hr. and re-suspending the pellet in fresh medium B.

| | Time of incubation at 30° (hr.) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Amino acid incorporation | | | |
| Medium not changed | 100 | 134 | 139 |
| Medium changed | 100 | 159 | 236 |
| Phosphorylation | | | |
| Medium not changed | 100 | 67·5 | 17·8 |
| Medium changed | 100 | 192 | 284 |

proteins (Fig. 6). More detailed analyses showed that the protein of highest specific radioactivity appears in a fraction sedimenting between 10 000*g* for 30 min. and 105 000*g* for 60 min. This fraction is also rich in phospholipid, RNA and succinoxidase activity (Table 5).

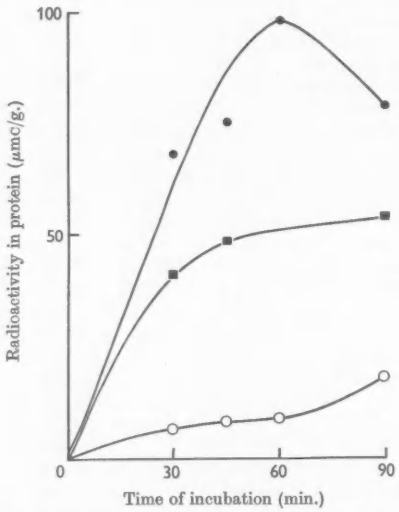


Fig. 6. Rate of incorporation of radioactive amino acids into protein of mitochondrial subfractions. ■, Intact mitochondria; ●, sediment after 60 min. at 105 000*g*; ○, supernatant after 60 min. at 105 000*g*. Mitochondria were incubated in medium B plus glucose and crude hexokinase and 1 μc of [$^{14}$C]valine/ml. At the times shown they were sedimented at 10 000*g* for 10 min. and disrupted with 0·2% (v/v) Triton X-100. Disrupted mitochondria were then centrifuged at 105 000*g* for 60 min.

### *Artifacts in studying mitochondrial incorporation of radioactive amino acids*

*Microsomal contamination.* The mitochondria used in these experiments have been well washed and probably have a low microsomal contamination, as indicated by their low RNA content. There was considerable variation in the amount of RNA found in the various preparations and the mean value was 8·85 ± 0·62 μg. of RNA-P/mg. of protein N (15 determinations), corresponding to approx. 1·4 mg. of RNA/100 mg. of protein. This variation was probably not due to the method of chemical fractionation, since phospholipid analyses gave more constant values [54·5 ± 0·31 μg. of phospholipid P/mg. of protein N (8 determinations) corresponding to approx. 21 mg. of phospholipid/100 mg. of protein]. It was also observed that the amount of RNA in the various preparations bore no obvious relationship to the amount of incorporation obtained in different experiments.

To confirm the efficiency of the washing procedure unwashed mitochondria were mixed with a radioactive microsomal preparation obtained from a rat previously injected with [$^{14}$C]algal protein hydrolysate. Four successive washings reduced the radioactivity in the protein from 50·3 to 1·4 μmc/g. Repeated washing also greatly reduced the amounts of RNA and glucose 6-phosphatase present, as would be expected from the removal of microsomal material. If the mitochondria at various stages of the washing procedure were incubated with radioactive amino acids, however, the

Table 5. *Biochemical properties of fractions obtained by disruption of mitochondria with Triton* X-100

Radioactivity in protein was determined after incubation of intact mitochondria for 1 hr. in medium B with 1·0 μc of [$^{14}$C]valine/ml. Mitochondria were centrifuged down, disrupted with 0·2% (v/v) Triton X-100 and sedimented at the speeds shown. Relative concentration is value/unit protein of fraction divided by value/unit protein in intact mitochondria. Values for mitochondria were 7·29 μg. of RNA-P and 53·9 μg. of phospholipid P/mg. of protein N, radioactivity 71·6 μmc/g./hr. and succinoxidase 328 μl. of $O_2$/hr./mg. of protein.

| Fraction ... ... ... | Sediment after 10 000*g* for 30 min. | Sediment after 105 000*g* for 60 min. | Final supernatant |
|---|---|---|---|
| | % of total amount in mitochondria | | |
| Phospholipid | 13·0 | 31·2 | 45·5 |
| RNA | 14·2 | 48·5 | 21·1 |
| Protein | 9·8 | 22·4 | 58·5 |
| Radioactivity in protein | 17·2 | 45·6 | 16·1 |
| | Relative concentration | | |
| Phospholipid | 1·32 | 1·40 | 0·78 |
| RNA | 1·45 | 2·07 | 0·36 |
| Succinoxidase | 0·58 | 1·14 | 0·01 |
| Radioactivity in protein | 1·73 | 2·05 | 0·28 |

Table 6. *Effect of ribonuclease on mitochondrial incorporation*

In Expts. 1–3 mitochondria were incubated in medium A plus 0·01 M-potassium phosphate, 0·07 M-tris–HCl, 8 mM-$MgSO_4$, 0·15 ml. of cell sap and 1·25 $\mu$c of algal protein hydrolysate/ml. at 37° for 1 hr. In Expt. 1, 20 $\mu$g. of Worthington ribonuclease/ml. was added. In Expt. 2, whole cell sap was treated with 100 $\mu$g. of Worthington ribonuclease/ml. for 15 min. at 37°; part of it was then boiled and used for Expt. 3. In Expt. 4 mitochondria were incubated for 1 hr. in medium B plus 0·33 $\mu$c of algal protein hydrolysate/ml., 0·2 $\mu$g. of highly purified ribonuclease (obtained from Dr Porter) was added/ml.

| Expt. no. | Additions to medium | Radioactivity in protein ($\mu$mc/g./hr.) | Change (%) |
|---|---|---|---|
| 1 | Whole cell sap | 62·0 | — |
| | Whole cell sap + ribonuclease | 74·0 | +19 |
| 2 | Whole cell sap | 100·0 | — |
| | Whole cell sap pretreated with ribonuclease | 124·0 | +24 |
| 3 | Boiled cell sap | 109·0 | — |
| | Cell sap pretreated with ribonuclease, then boiled | 125 | +14 |
| 4 | Synthetic amino acid mixture | 61 | — |
| | Synthetic amino acid mixture plus ribonuclease | 85 | +39 |

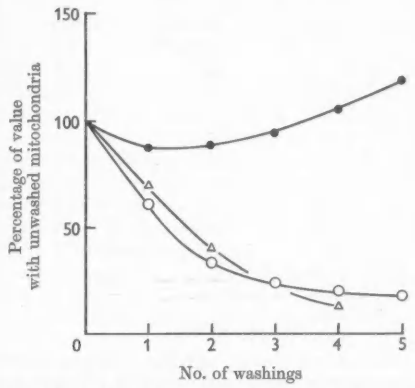


Fig. 7. Effect of washing on mitochondria. ●, Incorporation into protein after incubation for 2 hr. in medium B plus 0·33 $\mu$c of algal protein hydrolysate/ml.; ○, total RNA of suspension; △, total glucose 6-phosphatase of suspension. Unwashed mitochondrial suspension was obtained by centrifuging homogenate at 8000*g* for 10 min., after removal of nuclei and whole cells. They were washed by repeated sedimentation at 8000*g* for 10 min. After each washing, amino acid incorporation in medium B, RNA and glucose 6-phosphatase were determined. Initial values were: amino acid incorporation, 88·5 $\mu$mc/g./2 hr.; RNA, 126 $\mu$g. of RNA-P/ml. (36·2 $\mu$g. of RNA-P/mg. of protein N); glucose 6-phosphatase, 109 $\mu$moles of glucose 6-phosphate split/hr./ml.

incorporation observed was actually increased by repeated washing (Fig. 7).

Addition of amounts of ribonuclease sufficient to hydrolyse two to three times the amount of RNA present in the system had no inhibitory effect and in fact was slightly stimulatory (Table 6). It cannot be concluded that RNA is not required, however, since, although an excess of ribonuclease was added, only 25–30 % of the RNA present was destroyed by this treatment.

*Bacterial contamination.* Although there was no detectable contamination in the isolation and incubation media used, significant numbers of bacteria were found both in the mitochondrial suspensions and in the homogenates, although the numbers varied considerably from experiment to experiment. Removal of lobes of liver under sterile conditions suggested that most of the bacteria came from the liver itself and that very few were introduced during the preparation of the mitochondria.

It is unlikely that the amount of bacteria present contributed significantly to the observed amino acid incorporation, at least with incubation for 3 hr. First, there was no correlation between the amount of contamination and the incorporation (Table 7). Secondly, the incorporation was not reduced by incubation in the presence of penicillin and streptomycin (Table 7, Expt. 2). Thirdly, if the mitochondria were incubated in medium B for only 20 min. in the absence of an oxidizable substrate, the incorporation was reduced by 75 % (Fig. 8), the inhibition being irreversible. It is highly unlikely that in the presence of sucrose, inorganic phosphate, nucleotides, a complete mixture of amino acids and a high concentration of mitochondria, the contaminant bacteria would show a specific requirement for succinate. If the succinate is replaced by glucose, the incorporation is still greatly reduced, in parallel with the oxygen uptake (Fig. 9). The shape of the incorporation curve in the presence

Table 7. *Lack of correlation between amino acid incorporation and bacterial contamination in mitochondrial suspensions*

Mitochondria were incubated in medium B with 0·01 M-potassium succinate or 0·01 M-glucose in the presence of 0·33 $\mu$c of algal protein hydrolysate/ml.

| Expt. no. | $10^{-3}$ × No. of bacterial colonies: Colonies/ml. of reaction mixture | Colonies/g. of liver taken: Homogenate | Colonies/g. of liver taken: Mitochondria | Amino acid incorporation ($\mu$mc/g./hr.): Succinate | Amino acid incorporation ($\mu$mc/g./hr.): Glucose |
|---|---|---|---|---|---|
| 1 | 47·0 | — | — | 54·0 | — |
| 2 | 33·0 | 36·1 | 72·0 | 49·0 | 4·0 |
| | | | | 46·0* | — |
| 3 | 12·8 | 65·1 | 33·9 | 144·0 | — |
| 4 | 3·32 | 20·8 | 5·51 | 64·0 | — |
| 5 | 1·39 | — | 3·23 | 75·0* | 8·0* |
| 6 | 0·95 | 2·02 | 2·61 | 76·0 | 7·0 |

* 100 units of penicillin G and 60 $\mu$g. of streptomycin sulphate/ml.

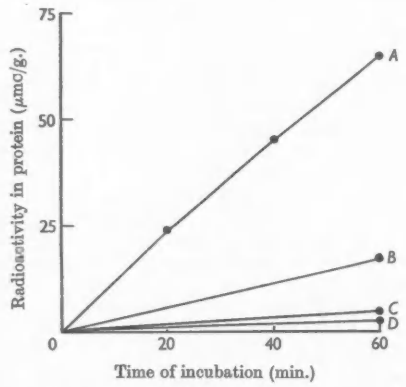


Fig. 8. Effect of omission of succinate from reaction mixture on amino acid incorporation. Mitochondria were incubated in medium B in the presence of 1·0 $\mu$c of [$^{14}$C]-valine/ml. Succinate was added at 0 min. (*A*), 20 min. (*B*), 40 min. (*C*) and at end of experiment (*D*).

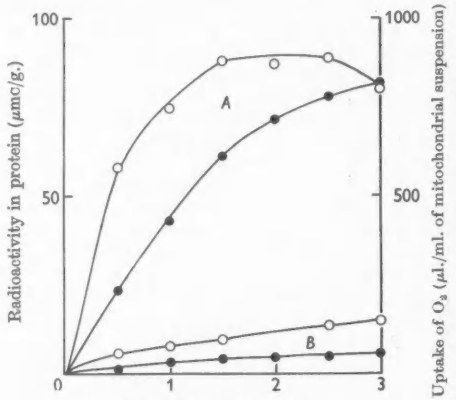


Fig. 9. Amino acid incorporation and oxygen uptake in the presence of succinate and glucose. ●, Oxygen uptake; ○, radioactivity in protein. *A*, Mitochondria incubated in medium B with succinate as energy source; *B*, 0·01 M-glucose instead of succinate. Media contained 0·33 $\mu$c of [$^{14}$C]algal protein hydrolysate/ml.

of succinate argues against bacterial contamination contributing to the system, since a definite plateau is obtained after incubation for 1½ hr., which would not be expected if the incorporation were due to bacteria. The low incorporation in the presence of glucose, however, appears to continue with time and it is possible that at least part of this is due to bacteria in the system, although even here there is no correlation between the incorporation observed and the amount of bacterial contamination (Table 7). The dependence of the system on oxidative phosphorylation, the inhibition by cyanide and 2:4-dinitrophenol (Table 3), the requirement for adenosine monophosphate (Table 3, Fig. 4), the response to added amino acids (Fig. 1) and the improvement in incorporation obtained by replenishing the medium (Table 4) provide additional arguments against the importance of bacterial contamination.

*Artifacts in labelling.* Treatment of radioactive mitochondrial protein, obtained after incubation in the presence of [$^{14}$C]algal protein hydrolysate, with performic acid (Peterson & Greenberg, 1952), ninhydrin (Peterson & Greenberg, 1952) or thioglycollic acid (Siekevitz, 1952) resulted in the release of less than 5% of the total radioactivity, indicating that the radioactive amino acids were not attached by S·S bonds or present as bound amino acid. Protein treated with 1-fluoro-2:4-dinitrobenzene (Sanger, 1945) was hydrolysed in

6·0 N-hydrochloric acid at 105° for 15 hr. in a sealed tube and the hydrolysate extracted with ether. About 1% of the radioactivity was extracted, indicating that only a small proportion of the radioactive amino acids were present as *N*-terminal residues.

## DISCUSSION

In studies on mitochondrial protein synthesis, particularly those requiring long periods of incubation, the problem of possible bacterial contamination takes on a special significance. First, any bacteria either present in the tissue taken, or introduced during the isolation procedure, will tend to become concentrated in the mitochondrial fraction, because of their similarity in size to mitochondria. Secondly, the media used for studying mitochondrial protein synthesis are ideal for bacterial growth, containing protein, amino acids, nucleotides, inorganic ions and oxidizable substrates. Thirdly, because of the much greater synthetic rate in micro-organisms, compared with the systems under study, a very small contamination could contribute significantly to the observed effect. In this work bacteria have indeed been detected in reasonable numbers, both in the final mitochondrial suspension and in the original liver taken. It has been possible to show, however, that this contamination does not contribute significantly to the observed incorporation for the times of incubation we have used. This agrees with the observation of Lamborg & Zamecnik (1960) with *Escherichia coli*, that, even with bacteria in the exponential phase of growth, 100 000 cells/ml. are needed before significant counts can be detected.

The mitochondrial and microsomal systems for amino acid incorporation differ in several respects. First, mitochondria do not require cell sap (Zamecnik & Keller, 1954) nor the pH 5 fraction obtained from it (Keller & Zamecnik, 1956), at least in our experiments. There is unfortunately some confusion in the literature about the importance of cell sap in the mitochondrial system. Greengard (1959) showed greater stimulation with whole cell sap than with boiled cell sap, as well as an effect with the pH 5 fraction. Korner (1959) found that cell sap improves the incorporation of radioactive leucine, but lowers that of radioactive tryptophan. Both these workers used conditions which would favour microsomal incorporation, however, and at least part of their results might have been due to microsomal contamination. McLean *et al.* (1958) reported that the pH 5 fraction can replace cell sap but in a later publication this group confirmed our observation (Reis *et al.* 1959) that cell sap is not inactivated by boiling (Bates, Craddock & Simpson, 1960). It is possible that intramitochondrial amino acid-activating enzymes are involved, although difficult to demonstrate satisfactorily (Table 2; Craddock & Simpson, 1960).

The second major difference between the two systems is in the effect of ribonuclease, the microsomes being inactivated by this enzyme (Allfrey, Daly & Mirsky, 1953; Zamecnik & Keller, 1954), whereas the mitochondria are either unaffected or actually stimulated (Table 6; McLean *et al.* 1958; Rendi, 1959*a*; Mager, 1960). The insensitivity to ribonuclease does not preclude RNA from the incorporation process, however, since only a part of the mitochondrial RNA is degraded by the enzyme, probably because of inaccessibility between enzyme and substrate. Certainly in two reports where amino acid incorporation was observed in fractions isolated from disrupted mitochondria (Rendi, 1959*a*; Kalf & Simpson, 1959) sensitivity to ribonuclease was observed.

The systems also differ in their stability. In our experiments incorporation proceeds for 1½–2 hr. if the medium is unchanged and for at least 3 hr. if it is replenished. Bates & Simpson (1959) have reported stability of calf-heart sarcosomes for 12 hr. In general the microsomal system is less stable, incorporation stopping after 15–30 min. (Simkin & Work, 1957*b*).

From these considerations it is unlikely that the incorporation observed in our experiments is due to microsomal contamination. For example, repeated washing of the mitochondria, although obviously removing the bulk of the microsomal contamination, actually increases the incorporation into the mitochondria. Other workers agree that the effect is not due to microsomes (McLean *et al.* 1958; Greengard, 1959), and Bates *et al.* (1960) have shown that microsomes could not synthesize cytochrome *c* under the conditions used to demonstrate mitochondrial synthesis of this protein. Mager (1960) has distinguished the two systems by the effect of chloramphenicol, the mitochondria being inhibited (see also Rendi, 1959*b*; Bates *et al.* 1960) and the microsomes not.

Since one can exclude microsomal contamination, studies on the role of RNA in the mitochondrial system might provide valuable evidence about the true significance of the small amounts of this nucleic acid found in washed mitochondria (see Harel, Jacob & Moulé, 1957; Novikoff, 1959). Disruption with Triton X-100 releases a certain amount of RNA into solution (Table 5), and this might include a mitochondrial soluble RNA. However, the bulk of the RNA remains attached to material rich in lipid and respiratory enzymes and sedimenting at 100 000*g*. By analogy with the results of other workers on disrupted mitochondria (for example, Cooper & Lehninger, 1956; Siekevitz & Watson, 1956, 1957; Watson & Siekevitz, 1956;

Abood & Alexander, 1957) this fraction is probably rich in mitochondrial membranes and also an important site for oxidative phosphorylation. The observation that the protein in this fraction is the most rapidly labelled (Table 5, Fig. 6) could therefore be attributed either to the membrane system or to ribonucleoprotein particles, as suggested by Rendi (1959*a*). The electron-microscope evidence in favour of the existence of intramitochondrial ribonucleoprotein particles is poor at the moment, however, and Siekevitz & Watson (1956) ascribed those particles present in fractions from disrupted mitochondria to microsomal contamination. More work is clearly required to establish the precise localization and function of RNA in the mitochondria.

It is difficult to distinguish the true requirements for mitochondrial amino acid incorporation from secondary effects on the efficiency of oxidative phosphorylation, and there is little doubt that many of the observed properties (e.g. dependence on inorganic phosphate, an oxidizable substrate, inhibition by cyanide, 2:4-dinitrophenol or anaerobic incubation) are primarily those of the phosphorylation system. For example, Bates *et al.* (1958) found that succinate, adenosine monophosphate, cytochrome *c* and inorganic phosphate could be partially replaced by adenosine diphosphate and creatine phosphate, showing that these substances are required primarily for ATP production and not for the incorporation process. If this is so, the general impression appears that the mitochondrion is highly autonomous with respect to protein synthesis, provided that it is incubated under conditions favouring continuous energy production. In fact, it bears more resemblance in its behaviour to an intact cell than to an isolated cell fragment. Since isolated mitochondria have been shown in the last decade to possess considerable structural and biochemical organization, it is not improbable that they also contain the complex equipment for protein synthesis.

## SUMMARY

1. Washed rat-liver mitochondria incorporate radioactive amino acids into their protein if incubated aerobically in the presence of an oxidizable substrate, inorganic phosphate, adenosine monophosphate, magnesium ions, diphosphopyridine nucleotide and a full complement of amino acids.

2. The system is slightly stimulated by ribonuclease and does not require cell sap or pH 5 enzyme. It has been shown that the observed incorporation is not due to microsomal contamination.

3. The incorporation is dependent upon the maintenance of oxidative phosphorylation by the mitochondria.

4. The optimum concentrations of adenosine monophosphate, magnesium ions, α-oxoglutarate, succinate and phosphate required for amino acid incorporation were determined.

5. Replenishment of the medium during incubation considerably improves the duration of incorporation.

6. Fractionation of radioactive mitochondria showed the highest rate of labelling to be in the protein of an insoluble fraction rich in ribonucleic acid and phospholipid. The soluble proteins, however, were poorly labelled.

7. Significant bacterial contamination was observed in the final mitochondrial fraction and in the livers originally taken. However, this contamination contributes negligible amounts to the observed incorporation at least during incubation for 3 hr.

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# The Regulation of Pathways of Glucose Catabolism in Maize Roots

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The operation of the pentose phosphate pathway in glucose catabolism, as demonstrated by low C-6/C-1 ratios (the yield of $^{14}CO_2$ from [6-$^{14}$C]-glucose relative to that from [1-$^{14}$C]glucose), can now be regarded as a feature of many plant tissues (Gibbs, 1959). On the other hand, the internal and external conditions which determine the extent of its activity have received little attention. In the work described here, the action of oxidizing agents on the yield of $^{14}CO_2$, when maize root-tips were provided with glucose specifically labelled in various carbon atoms, has been examined and compared with nitrite as a likely natural electron acceptor, and with 2:4-dinitrophenol as an uncoupling agent.

Maize root-tips, in common with other young developing plant tissues (Gibbs & Beevers, 1955), show a C-6/C-1 ratio of unity (Beevers & Gibbs, 1954), suggesting that hexose breakdown proceeds primarily through the classical pathways of glycolysis and the tricarboxylic acid cycle. However, Beevers (1956) has shown that such tissues break down labelled gluconate, ribose, xylose and sedoheptulose in air and under nitrogen to give products with the radiochemical distribution expected from the operation of the pentose phosphate pathway, so that they must possess the necessary enzymic equipment, which appears to be inoperative when glucose is the substrate at this stage. Any induction of the alternative oxidation pathway should therefore be recognized readily by a decrease in the natural C-6/C-1 ratio.

The stimulation of the oxygen consumption and carbon dioxide production of plant tissues by artificial hydrogen acceptors, especially by methylene blue (Turner, 1940), has been recognized for some time (James, 1953), and recent work with carrot disks (ap Rees & Beevers, 1960) has shown

that this is accompanied by a vigorous increase in the release of C-1 of glucose. In work with carrot tissue (ap Rees & Beevers, 1960), liver slices (Cahill, Hastings, Ashmore & Zottu, 1958), erythrocytes (Brin & Yonemoto, 1958), corneal epithelium (Kinoshita, 1957) and ascites-tumour cells (Wenner, 1959), the effects of such reagents have been interpreted as showing that the supply of triphosphopyridine nucleotide is a major factor in controlling the activity of the pentose phosphate pathway. A similar demonstration was sought in young maize roots, by treating them with three oxidizing agents under both aerobic and anaerobic conditions.

The possibility that natural oxidants for reduced triphosphopyridine nucleotide present within tissues elicit the participation of the pentose phosphate pathway has been raised. Thus the assimilation of ammonia by yeast cells (Holzer & Witt, 1960) and possibly the synthesis of fatty acids in lactating rat mammary tissue (Abraham, Cady & Chaikoff, 1957; McLean, 1960) are activities which may be coupled with the pathway. The reduction of inorganic nitrogen in plant tissues suggests another likely activity, and the changes induced in the pattern of metabolism during nitrite assimilation by maize roots have now been examined.

A preliminary account of this work has been presented (Butt & Beevers, 1960).

## EXPERIMENTAL

Maize grain (hybrid variety Wf 9 × 38–11) was soaked under water overnight for 16 hr., and allowed to germinate between moist absorbent paper for 24 hr. at 30°. The roots were then up to 2 cm. long; the first 1 cm. segment from the apex was cut as exactly as possible. The segments were washed thoroughly with deionized water and used within 2 hr. after excision. In each vessel 20 root-tips were generally used; the mean fresh weight for six samples was 0·217 ± 0·009 g.

Uniformly labelled glucose, [1-$^{14}$C]-, [2-$^{14}$C]- and [6-$^{14}$C]-glucose were commercial products. The sample of [3:4-$^{14}C_2$]-glucose had been prepared by exposing castor beans to $^{14}CO_2$ and contained at least 90% of the radioactivity in C-3 and C-4 (ap Rees & Beevers, 1960).

*Methods.* The root-tips were incubated in Warburg vessels with $KH_2PO_4$–$Na_2HPO_4$ (0·067 M) or water at 25°. Measurements of gas exchange were carried out by standard manometric techniques. Production of $CO_2$ was determined by the direct method of Warburg; the flask constants were adjusted to allow for $CO_2$ retention in the buffers used (Umbreit, Burris & Stauffer, 1957). In experiments with labelled substrate, 2 μmoles of glucose, containing 0·05–0·006 μc of $^{14}$C, were supplied in a total volume of 2·5 ml.

The $^{14}CO_2$ released by the tissues was absorbed by KOH in the centre wells. At intervals, usually after incubation for 3 and 6 hr., the KOH was removed quantitatively and replaced by fresh alkali. The carbonate present in each sample of KOH was precipitated as $BaCO_3$ and the $^{14}$C content measured by counting in a windowless gas-flow counter. C-6/C-1 ratios are calculated as described by Beevers & Gibbs (1954).

Glucose uptake was estimated in replicate vessels or in separate experiments by taking out samples (0·1–0·2 ml.) at suitable intervals and determining their radioactivity after drying gently on a nickel planchet.

## RESULTS

### *Effect of artificial electron acceptors*

*Rate of respiration.* After the root-tips had been excised, their respiratory rate showed a steady decline over some hours. As the gas measurements were started within 2 hr. after excision, they reflect this decline. In one experiment 20 root-tips absorbed 155, 108, 102, 97, 99 and 97 μl. of $O_2$ in successive periods of 1 hr., during which $CO_2$ production fell from 122 to 93 μl., so that the R.Q. showed little change with an average of 0·95 ± 0·01.

The effects of the three artificial electron acceptors, methylene blue, phenazine methosulphate and triphenyltetrazolium chloride at concentrations giving maximum stimulations are shown in Table 1. Methylene blue (2 mM) stimulated $O_2$ uptake and $CO_2$ output equally without any effect on the R.Q.; the stimulations, though small, were reproduced in three experiments. Phenazine methosulphate, at 1 mM and at lower concentrations, increased the rate of $CO_2$ production rather more than that of $O_2$ uptake so that the R.Q. was in all cases increased to nearly 1·0. Triphenyltetrazolium chloride, applied at pH 7·3 to increase its

Table 1. *Effect of artificial electron acceptors on the respiration of maize root-tips*

Twenty maize root-tips were incubated in $KH_2PO_4$–$Na_2HPO_4$ buffer at 25° for 3 hr. Total volume, 2·5 ml.

| Additions | pH | $O_2$ uptake (μl./hr.) | $CO_2$ production (μl./hr.) | R.Q. |
|---|---|---|---|---|
| None | 6·0 | 107 | 83 | 0·77 |
| 2 mM-Methylene blue | 6·0 | 117 | 91 | 0·78 |
| None | 6·0 | 144 | 109 | 0·76 |
| 1 mM-Phenazine methosulphate | 6·0 | 171 | 163 | 0·95 |
| None | 7·3 | 124 | 105 | 0·85 |
| 8 mM-Triphenyltetrazolium chloride | 7·3 | 99 | 164 | 1·67 |

effectiveness (Marrè & Arrigoni, 1954), gave a heavy red precipitate in the tissue. At 8 mM, as shown in Table 1, $O_2$ uptake was lowered but the $CO_2$ output was considerably stimulated, so that the R.Q. rose as high as 1·67; higher concentrations reduced the $O_2$ consumption still further. Over a further period of 3 hr., these differences were sustained with each reagent; the R.Q. values remained almost constant despite falls in the rates of gas exchange.

*Glucose uptake.* During successive periods of 3 hr. the rate of glucose uptake into untreated roots remained constant, although a large proportion of the applied glucose was absorbed. Glucose uptake was considerably lowered by phenazine methosulphate and triphenyltetrazolium chloride and less so by methylene blue (Table 2).

*Output of* $^{14}CO_2$. When only a small fraction of the glucose absorbed by the untreated roots was released as $CO_2$, the C-6/C-1 ratio was close to 1 in this material (Table 3). The result of adding each of the artificial electron acceptors was a striking (two- to three-fold) stimulation in the rate of $^{14}CO_2$ release from [1-$^{14}$C]glucose, and this in spite of the fact that glucose uptake was curtailed. The release of C-1 in the presence of phenazine methosulphate and triphenyltetrazolium chloride was such that it represented an almost complete utilization of the [1-$^{14}$C]glucose which had been absorbed. In addition these reagents appreciably suppressed the release of C-6 as $CO_2$ so that the resulting C-6/C-1 ratios were extremely low (Table 3). In the presence

Table 2. *Effect of artificial electron acceptors on glucose uptake*

Twenty maize root-tips were incubated with 2 $\mu$moles of uniformly labelled [$^{14}$C]glucose in buffer, as in Table 1. Total volume, 2·5 ml. The residual glucose was measured after 3 hr. and 6 hr.

| Additions | pH | Uptake (%) 3 hr. | 6 hr. |
|---|---|---|---|
| None | 6·0 | 28 | 60 |
| 2 mM-Methylene blue | 6·0 | 20 | 38 |
| None | 6·0 | 31 | 73 |
| 1 mM-Phenazine methosulphate | 6·0 | 25 | 25 |
| None | 7·3 | 18 | 40 |
| 8 mM-Triphenyltetrazolium chloride | 7·3 | 5 | 6 |

Table 3. *Effect of artificial electron acceptors on the release of* $^{14}CO_2$ *from* [1-$^{14}$C]- *and* [6-$^{14}$C]-*glucose*

Conditions were as in Table 2.

| Additions | Period (hr.) | Conversion of applied glucose into $^{14}CO_2$ (%) [1-$^{14}$C] | [6-$^{14}$C] | C-6/C-1 |
|---|---|---|---|---|
| None | 0–3 | 2·5 | 2·2 | 0·88 |
| | 3–6 | 4·9 | 4·5 | 0·92 |
| 2 mM-Methylene blue | 0–3 | 7·5 | 2·1 | 0·28 |
| | 3–6 | 10·6 | 4·2 | 0·39 |
| None | 0–3 | 1·4 | 1·4 | 1·00 |
| | 3–6 | 3·5 | 3·0 | 0·86 |
| 1 mM-Phenazine methosulphate | 0–3 | 8·5 | 1·0 | 0·12 |
| | 3–6 | 12·1 | 2·8 | 0·23 |
| None | 0–3·5 | 1·0 | 0·9 | 0·90 |
| | 3·5–7 | 2·7 | 2·1 | 0·78 |
| 8 mM-Triphenyltetrazolium chloride | 0–3·5 | 2·3 | 0·3 | 0·13 |
| | 3·5–7 | 4·4 | 0·9 | 0·20 |

Table 4. *Effect of artificial electron acceptors on the release of* $^{14}CO_2$ *from specifically labelled glucose*

Thirty maize root-tips were incubated under the conditions in Table 2.

| Additions | Period (hr.) | $O_2$ uptake ($\mu$l./hr.) | Glucose uptake (%) | Conversion of applied glucose into $^{14}CO_2$ (%) [1-$^{14}$C] | [2-$^{14}$C] | [3:4-$^{14}C_2$] | [6-$^{14}$C] |
|---|---|---|---|---|---|---|---|
| None | 0–3 | 166 | 31 | 2·0 | 0·7 | 5·1 | 1·8 |
| | 3–6 | 163 | 31 | 3·6 | 2·6 | 10·2 | 3·2 |
| 2 mM-Methylene blue | 0–3 | 193 | 21 | 4·4 | 1·8 | 6·1 | 1·5 |
| | 3–6 | 177 | 14 | 5·3 | 3·9 | 8·9 | 2·2 |
| 1 mM-Phenazine methosulphate | 0–3 | 218 | 18 | 10·8 | 4·4 | 3·0 | 0·8 |
| | 3–6 | 205 | 13 | 13·3 | 8·3 | 7·6 | 2·3 |

of methylene blue, the release of C-6 was only slightly affected so that the C-6/C-1 ratios were not so severely decreased. When the $^{14}CO_2$ release from [2-$^{14}$C]glucose was measured (Table 4) it became evident that phenazine methosulphate and methylene blue also had striking stimulatory effects on the release of C-2 as $CO_2$. During the 3–6 hr. period in the phenazine methosulphate experiments, the release of C-2 was almost as great as in the preceding 3 hr., although that of C-1 was declining as it reached completion. This increase in release of C-2 contrasts with the severe decrease in $^{14}CO_2$ production from [3:4-$^{14}C_2$]glucose with phenazine methosulphate; methylene blue had little effect on the release of C-3:4.

*Effect of artificial electron acceptors under nitrogen*

Both methylene blue and tetrazolium were reduced when they were incubated with maize roots under $N_2$; triphenyltetrazolium chloride gave a heavy red precipitate in the tissue and methylene blue was decolorized both in the tissue and in the ambient solution. The reduction was accompanied by a stimulation of $CO_2$ production (Table 5). This was sustained while oxidizing agent was available, but, with 2 mM-methylene blue, fermentation returned to the control rate after 4 hr., when the dye had been completely decolorized.

Neither of the oxidants, at the concentrations employed, affected seriously the release of $^{14}CO_2$ from [3:4-$^{14}C_2$]glucose nor the very slight and barely significant release of C-6. With each, however, the rate of release of C-1 was clearly increased and the threefold increase observed with 10 mM-methylene blue was sufficient to account for the extra $CO_2$ production which it induced. The lower stimulation of C-1 release with 2 mM-methylene blue might be anticipated from the fact that reduction was complete before the end of the incubation period. The glucose uptake was sufficient in all cases to avoid any limitation of radiocarbon supply.

In these experiments, the [3:4-$^{14}C_2$]glucose was applied in greater concentration than the other sugars but this does not affect their quantitative interpretation, since parallel experiments showed that the proportions of applied glucose absorbed and of $^{14}CO_2$ produced were unaffected by this difference.

*Effect of 2:4-dinitrophenol*

The pattern of radiocarbon release with the artificial electron acceptors was contrasted with the effect of an uncoupling agent, 2:4-dinitrophenol. Beevers (1953) observed that dinitrophenol stimulation of $O_2$ uptake may be accompanied by a greater stimulation of $CO_2$ production with a consequent rise in R.Q. At 0·05 mM, $O_2$ uptake was found here to be stimulated by 15% and $CO_2$ production by 70% over 3 hr., so that the R.Q.

Table 5. *Effect of artificial electron acceptors on fermentation and the conversion of specifically labelled glucose into $^{14}CO_2$ by maize root-tips under nitrogen*

Fifty maize root-tips were incubated in $KH_2PO_4$–$Na_2HPO_4$ buffer (0·067 M) at 25° under $N_2$. [1-$^{14}$C]Glucose or [6-$^{14}$C]glucose (2 $\mu$moles), or 3·3 $\mu$moles of [3:4-$^{14}C_2$]glucose were added. Total volume, 2·5 ml.; incubation period, 4 hr.

| Additions | pH | $CO_2$ production ($\mu$l./hr.) | Glucose uptake (%) | Conversion of applied glucose into $^{14}CO_2$ (%) | | |
|---|---|---|---|---|---|---|
| | | | | [1-$^{14}$C] | [3:4-$^{14}C_2$] | [6-$^{14}$C] |
| None | 6·0 | 115 | 21 | 0·5 | 8·4 | 0·0 |
| 10 mM-Methylene blue | 6·0 | 130 | 20 | 1·8 | 7·7 | 0·0 |
| None | 6·0 | 145 | — | 1·6 | 13·3 | 0·1 |
| 2 mM-Methylene blue | 6·0 | 167 | — | 2·4 | 11·9 | 0·1 |
| None | 7·3 | 124 | 11 | 0·4 | 8·2 | 0·1 |
| 8 mM-Triphenyltetrazolium chloride | 7·3 | 166 | 38 | 0·8 | 8·2 | 0·1 |

Table 6. *Effect of 2:4-dinitrophenol on the release of $^{14}CO_2$ from specifically labelled glucose*

Thirty maize root-tips were incubated under the conditions in Table 2, at pH 6·0.

| Additions | Period (hr.) | Glucose uptake (%) | Conversion of applied glucose into $^{14}CO_2$ (%) | | | |
|---|---|---|---|---|---|---|
| | | | [1-$^{14}$C] | [2-$^{14}$C] | [3:4-$^{14}C_2$] | [6-$^{14}$C] |
| None | 0–3 | 31 | 2·0 | 0·7 | 5·1 | 1·8 |
| | 3–6 | 31 | 8·6 | 2·6 | 10·2 | 8·2 |
| 0·05 mM-Dinitrophenol | 0–3 | 19 | 1·5 | 2·5 | 9·8 | 1·0 |
| | 3–6 | 26 | 5·4 | 6·0 | 15·6 | 3·6 |

rose from 0·73 to 1·08; in the subsequent 3 hr., both stimulation and R.Q. declined.

Despite the reduction in absorption of [$^{14}$C]-glucose (Table 6), the release of C-3:4 and of C-2 was considerably increased. By contrast, the release of C-1 and C-6 was less than that in the controls; a slight but significant fall in the C-6/C-1 ratio was observed.

*Effect of sodium nitrite*

The assimilation and reduction of nitrite in plant tissues is accompanied by a stimulation of $CO_2$ production with relatively little effect on the $O_2$ uptake (Willis & Yemm, 1955; Syrett, 1955). Similar effects of nitrite assimilation on the metabolism of maize root-tips were observed when nitrite was supplied in unbuffered solution. With 5 mM-$NaNO_2$, the $O_2$ uptakes of control and nitrite-treated roots were 121 and 123 $\mu$l./hr. Outputs of $CO_2$ were 122 and 145 $\mu$l./hr. to give R.Q. values of 0·93 and 1·18 respectively over 3 hr. The stimulation of $CO_2$ output declined in the subsequent 3 hr. period. The uptake of glucose was virtually unaffected by treatment with nitrite.

Table 7 shows that nitrite induced very different effects on the contributions of the various carbons of glucose to the respired $CO_2$. Thus while the release of C-6 was reduced, that of C-2 and particularly of C-1 was strikingly increased.

## DISCUSSION

The three artificial electron acceptors employed here show a useful range of biochemical and metabolic activity. Phenazine methosulphate oxidizes reduced pyridine nucleotide directly and non-enzymically, but methylene blue and triphenyltetrazolium chloride are effective only through diaphorase action. Further, although the first two are each auto-oxidizable after reduction, the red formazan precipitate derived from tetrazolium reduction cannot be reoxidized by air. These differences are reflected in their metabolic effects, for, although each induced a stimulation of carbon dioxide production, methylene blue and phenazine methosulphate acted as alternative electron carriers to oxygen and in fact stimulated oxygen uptake; triphenyltetrazolium chloride decreased oxygen consumption by the tissue. However, treatment with each reagent strikingly reduced the C-6/C-1 ratio primarily by increasing the contribution of C-1 to the respired carbon dioxide. This suggests that the operation of the pentose phosphate pathway was increased more than glucose catabolism through glycolysis and the tricarboxylic acid cycle.

Support for this interpretation comes from the fact that the release of C-2 as well as that of C-1 was increased by treatment with methylene blue and phenazine methosulphate whereas that of C-3:4 was actually depressed. In the presence of phenazine methosulphate, the contributions to respired carbon dioxide were C-1 > C-2 > C-3; precisely the reverse of those predicted from a sequence comprising only the Embden–Meyerhof–Parnas pathway and the tricarboxylic acid cycle. These results are those predicted if the glucose units formed during the passage through the pentose phosphate pathway (with the original C-2 now primarily in position 1) were recycled in this sequence. The reduction in the fraction of glucose catabolized through the Embden–Meyerhoff–Parnas sequence as a result of removal of restraint at the oxidative steps of the pentose phosphate pathway would decrease still further the contribution of C-3:4 to the respired carbon dioxide in short-term experiments.

It is clear of course from the data on glucose uptake and release of $^{14}CO_2$ that the effects of the three oxidants are not identical, and are not limited to facilitating the oxidation of reduced TPN (Smith, 1952; Novikoff, 1959; Stenlid, 1950; Gaur & Beevers, 1959). Nevertheless it seems reasonable to conclude that the positive action of each in increasing the contribution of C-1 and C-2 to respired carbon dioxide is due to this common feature, and this implies that the participation of the pentose phosphate pathway in this tissue is limited by the capacity for oxidation of reduced TPN.

The effects of nitrite on the release of individual carbons as carbon dioxide (Table 7) are strikingly

Table 7. *Effect of sodium nitrite on the release of $^{14}CO_2$ from specifically labelled glucose*

Thirty maize root-tips were incubated in water at 25° with 2 $\mu$moles of labelled glucose. Total volume, 2·5 ml.

| Additions | Period (hr.) | Glucose uptake (%) | Conversion of applied glucose into $^{14}CO_2$ (%) [1-$^{14}$C] | [2-$^{14}$C] | [6-$^{14}$C] | C-6/C-1 |
|---|---|---|---|---|---|---|
| None | 0–3 | 30 | 3·2 | 1·1 | 4·0 | 1·25 |
| | 3–6 | 43 | 6·0 | 3·3 | 6·5 | 1·08 |
| 5 mM-Sodium nitrite | 0–3 | 33 | 7·2 | 3·1 | 2·6 | 0·36 |
| | 3–6 | 38 | 19·3 | 10·0 | 5·7 | 0·30 |

similar to those induced by the oxidants, particularly by methylene blue. This suggests that the carbon dioxide burst accompanying nitrite treatment arises from decarboxylation in the pentose phosphate pathway, which, we presume is stimulated as reduced TPN is reoxidized during nitrite reduction. Such a reduction accompanied by the same effects on carbon dioxide output has been demonstrated in barley roots (Yemm & Willis, 1956) and *Ankistrodesmus* (Kessler, 1953) and the requisite enzymes have been isolated from higher plants (Nason, Abraham & Averbach, 1954). The sustained release of C-6 and the unimpaired uptake of glucose in the presence of nitrite suggests that glycolysis and the tricarboxylic acid cycle are not adversely affected.

The effect of stimulation of glycolysis and the acid cycle is shown in the response to dinitrophenol (Table 6). Results from pyruvate feeding experiments (Neal & Beevers, 1960) suggest that the release of C-3:4 should be immediately accelerated with C-2 and C-1:6 successively less affected. In spite of the reduction of glucose uptake due to treatment with dinitrophenol the anticipated stimulatory effects on the release of C-3:4 and of C-2 are indeed observed. The conclusion from these results is similar to that drawn from experiments with carrot, which, however, differs from the tissue used here in that the pentose phosphate pathway contributes noticeably to the respiration of the untreated material (ap Rees & Beevers, 1960). The results with dinitrophenol form a useful contrast with the effects of the artificial oxidants and nitrite.

Finally, an interesting problem is raised by the pattern of $^{14}CO_2$ release in the controls in Tables 4 and 7. Although $^{14}CO_2$ production from [1-$^{14}$C]- and [6-$^{14}$C]-glucose were about equal, that from [2-$^{14}$C]glucose was less than either; according to the foregoing arguments, it should be greater if the pentose phosphate pathway is inoperative. In each case, the release of C-3:4 was greatest. Humphreys & Duggar (1959) have reported similar observations. The results suggest that in this tissue, although the C-6/C-1 ratio is unity, some glucose breakdown was occurring by the pentose phosphate sequence. A likely explanation of this effect is that the excess of C-1 produced by this sequence is balanced by the release of C-6 during pentosan formation from glucose (Slater & Beevers, 1958). Equilibration between C-1 and C-6 of glucose (Shibko & Edelman, 1957) would also tend to increase the ratios towards unity (Axelrod & Beevers, 1956). These results emphasize again the desirability of providing supporting evidence from the respiration of glucose specifically labelled in other carbon atoms to sustain even qualitative deductions about pathways of breakdown.

## SUMMARY

1. The effects of artificial electron acceptors on the respiration of maize root-tips and on their release of $^{14}CO_2$ from specifically labelled glucose in air and under nitrogen have been investigated.

2. Methylene blue, phenazine methosulphate and triphenyltetrazolium chloride each stimulated the production of carbon dioxide, with preferential release of $^{14}CO_2$ from [1-$^{14}$C]glucose. In air, $^{14}CO_2$ release from [2-$^{14}$C]glucose was also stimulated.

3. By comparison, 2:4-dinitrophenol increased the release of $^{14}CO_2$ from [3:4-$^{14}$C]- and, rather less, from [2-$^{14}$C]-glucose, but decreased that from [1-$^{14}$C]glucose.

4. Treatment with nitrite gave effects similar to those with the artificial electron acceptors.

5. It is suggested that the activity of the pentose phosphate pathway in this tissue is limited by the supply of oxidized triphosphopyridine nucleotide, and that nitrite reduction is coupled with its operation.

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# The Effect of Pituitary Growth Hormone on Phospholipid Synthesis

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Amongst the many metabolic effects described in rats treated with growth hormone are the marked modifications in fat metabolism such as an increased rate of fatty acid oxidation (Greenbaum & McLean, 1953*a*) and a decreased rate of fatty acid synthesis (Greenbaum & Glascock, 1957). Phospholipid synthesis is also affected, although previous studies on the effect of the hormone on the rate of phospholipid synthesis in the livers of rats have led to some discordant results. Greenbaum, Graymore & Slater (1957) have reported a substantial increase in the rate of incorporation of injected [$^{32}$P]orthophosphate into the liver phospholipids of growth-hormone-treated animals, whereas Greenbaum & Glascock (1957) found a threefold decrease in the rate of incorporation of $^{14}$C from [1-$^{14}$C]acetate into phospholipids of similarly treated animals. It appeared desirable to investigate this discrepancy more closely and to attempt to obtain further information on the nature of the action of the hormone which results in the lower incorporation rate of $^{14}$C.

To this end the decreased rate of $^{14}$C incorporation into phospholipid has been examined in more detail in an attempt to establish at which steps in the pathway of biosynthesis of phospholipids this decrease occurred and how far it is explicable in terms of isotope dilution caused by the appearance of larger quantities of intermediates of the synthetic pathway in the livers of growth-hormone-treated animals. By use of [$^{14}$C]acetate and [$^{14}$C]-palmitate as precursors it has been established that one of the spans affected by the hormone is acetate → long-chain acyl-coenzyme A. A second factor leading to the decrease in radioactive labelling is tentatively identified as a dilution at the level of diglyceride, which appears to be present in greater quantities in the livers of growth-hormone-treated rats than in control livers.

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## METHODS

*Animals.* Adult female rats of the hooded Norwegian strain aged about 3–4 months and weighing 170–200 g. were fed *ad lib.* on diet 41 of Bruce & Parkes (1946).

*Growth hormone.* A twice-recrystallized preparation of anterior-pituitary growth hormone prepared from ox pituitaries by the method of Wilhelmi (1955) was used. Rats were injected with 1 mg. of the hormone (in 1 ml. of water) and killed 6 hr. later. This time was chosen as it had been shown by Greenbaum & Glascock (1957) that there is a considerable inhibition of lipogenesis in the livers of growth-hormone-treated rats at this time-interval after the injection. Controls were injected with 1 ml. of 0·9% sodium chloride soln. After killing, the livers were rapidly removed, rinsed and placed in ice-cold Ringer bicarbonate (Umbreit, Burris & Stauffer, 1945). In all experiments in which incorporation of the two precursors has been compared, the liver slices used were always taken from the same liver.

*Incubation and isolation procedures.* For some of the experiments on the incorporation of acetate and palmitate into fatty acids, phospholipids and neutral fats, 400 mg. of slices were cut from the livers with a Stadie–Riggs (1944) microtome and incubated in 5 ml. of Krebs–Ringer bicarbonate in the presence of either [1-$^{14}$C]acetate or [1-$^{14}$C]palmitate at a final concentration of 0·1 $\mu$c/ml. The [1-$^{14}$C]acetate had a specific activity of 4 mc/m-mole and the [1-$^{14}$C]palmitate 2 mc/m-mole. Both substances were obtained from The Radiochemical Centre, Amersham, Bucks. No carrier was added. The flasks were gassed with $CO_2 + O_2$ (5:95) and incubated at 37° for 3 hr. At the end of this time the slices were collected by centrifuging, washed twice with ice-cold 0·9% sodium chloride soln. and then suspended and homogenized in 5 ml. of 5% trichloroacetic acid in an all-glass Potter–Elvehjem (1936) homogenizer. After centrifuging, the residue was again suspended in 5 ml. of 5% trichloroacetic acid and the extracts were discarded.

The residue was then suspended in 1 ml. of water, 5 ml. of absolute ethanol was added and the whole stood overnight at room temperature. The extract was then separated by centrifuging and the residue extracted twice with ethanol–ether (3:1) and once with ether. The solvents were removed by evaporation and the residue was extracted with light petroleum (4 × 3 ml.). From the combined extracts the phospholipids were separated as their magnesium complexes and purified by redissolving in 0·6 ml. of chloroform and precipitating again with acetone (Greenbaum & Glascock, 1957). The phospholipid-free extract was used as a source of the neutral lipids, the only purification procedure being the removal of the labelled palmitic acid in the experiments on the incorporation of palmitate into neutral lipids. For this purification the solution of neutral lipids in acetone was evaporated to dryness, the residue dissolved in light petroleum (b.p. 40–60°) and shaken in a separating funnel with cold aqueous 0·02 N-potassium hydroxide solution. This procedure was repeated twice and appeared to remove adequately any free palmitic acid.

For the experiments on the conversion of palmitic acid into palmitoyl-coenzyme A (CoA), liver homogenates (1 g. of liver with 4 ml. of 0·9% sodium chloride soln.) were used. A portion (1 ml.) of the homogenate was incubated in 4 ml. of water containing 7·5 m-moles of hydroxylamine hydrochloride (freshly neutralized to pH 7·4 by the addition of ammonia); 154 $\mu$moles of sodium chloride and 0·025 $\mu$mole of [1-$^{14}$C]palmitate ($1{\cdot}23 \times 10^6$ counts/min.). After incubating for 2 hr. at 37°, 5 ml. of 10% perchloric acid was added to each flask, the whole then centrifuged, and the liquid phase removed with a capillary pipette. The residue was washed twice with portions (5 ml.) of 5% perchloric acid and extracted three times with portions (3 ml.) of the reagent A of Hill (1947) diluted 1:100 with methanol. Unchanged palmitic acid was separated from the ferric hydroxamate by repeatedly shaking the methanolic solution with heptane according to the method of Kornberg & Pricer (1953). Samples of the methanolic solution were plated and counted as described below.

For the experiments on the conversion of phosphatidic acid into diglyceride, 1:20 liver homogenates in 0·05 M-sodium maleate buffer, pH 6·4 (Smith, Weiss & Kennedy, 1957), were prepared. The reaction flasks contained 200 $\mu$moles of sodium maleate buffer, pH 6·4, 14 $\mu$moles of phosphatidic acid and homogenate equivalent to 200 mg. of rat liver. Incubation was for 1 hr. at 37° and the reaction was then stopped by the addition of 4 ml. of 10% perchloric acid. Inorganic phosphate was determined on samples of the supernatant after centrifuging and the figures so obtained were corrected for the blank values obtained in the flasks incubated without substrate (Smith *et al.* 1957).

For measurement of the rate of incorporation of [$^{32}$P]-orthophosphate into phosphatidic acids, liver mitochondria (in 0·25 M-sucrose) prepared by the procedure of Hogeboom, Schneider & Pallade (1948) were used. The mitochondria were incubated at 37° for 1 hr. in a medium identical with that described by Kennedy (1953) except that adenosine diphosphate (ADP) was used in place of adenosine monophosphate (AMP) and no glycerol was added. After incubation the phosphatides were extracted and purified as described by Kennedy (1953) and plated and counted as described below.

In the experiments on the incorporation of phosphorylcholine into phospholipids, the liver preparation was a particulate fraction of a sucrose homogenate prepared according to Kennedy & Weiss (1956). This fraction includes particles smaller than mitochondria. Each flask contained 100 $\mu$moles of sodium phosphate buffer, pH 7·4, 10 $\mu$moles of adenosine triphosphate (ATP), 1·5 $\mu$moles of cytidine triphosphate, 20 $\mu$moles of magnesium chloride, [$^{32}$P]phosphorylcholine in amounts between 4 and 20 $\mu$moles and 'liver particles' derived from 0·4 g. of liver. ATP (1·5 $\mu$moles) and 0·03 $\mu$mole of cytidine triphosphate were added every 12 min. during the course of the experiment. Incubation was for 1 hr. at 37°. After incubation the phospholipids were extracted and purified according to Kennedy & Weiss (1956) and counted as described below.

For the experiment on the labelling and chromatographic separation of the neutral lipids, the combined livers of four experimental or four control rats were used. The livers were rapidly weighed, chopped finely with scissors and finally reduced to a brei by pressing through the nozzle of a plastic syringe. The brei thus obtained was incubated for 3 hr. at 37° as described above for slices except that all components were increased in proportion to the weight of the liver. The substrates used were [1-$^{14}$C]acetate and [$^{32}$P]orthophosphate, present together. After incubation the lipids were extracted as before. The phospholipids were separated by precipitation with cold acetone and dried under vacuum. They were then extracted with ethanol to yield two fractions: the ethanol-soluble (lecithins) and ethanol-insoluble (kephalins). The acetone solution containing neutral lipids was evaporated to dryness at room temperature and the residue extracted with *n*-hexane (b.p. 67–70°). The residue from this extraction was set aside. The clear hexane solution was applied to a silicic acid column (11 cm. × 3·7 cm.) and the separation of neutral lipids carried out as described by Barron & Hanahan (1958). Fractions (15 ml.) were collected, evaporated to dryness and weighed and counted; the volume plated was adjusted so that the weight of lipid on the planchet did not exceed 1·5 mg. The fractions containing the triglycerides and free fatty acids were combined and dissolved in light petroleum (b.p. 40–60°), and the triglycerides separated from the free fatty acids by the method of Borgström (1952).

### *Chemical methods*

*Preparation of phosphatidic acids.* The starting material for this preparation was lecithin prepared by the method of Hanahan, Turner & Jayko (1951) with alumina columns. Each 50 ml. fraction was analysed for phosphorus and choline (Glick, 1944) and only those fractions with a phosphorus to choline ratio of 1 were used for the next step. The purified lecithin was incubated for 2 hr. with carrot chloroplasts at 25°, in the presence of ether, as described by Kates (1955). The sodium salts of the phosphatidic acids were purified by repeated precipitation from ether solution by the addition of acetone and finally dried under vacuum. An aqueous suspension was prepared by the addition of water to the sodium salts of the phosphatidic acids and the suspension homogenized in a tight-fitting Potter–Elvehjem (1936) homogenizer until a very fine suspension was obtained.

*Preparation of* [$^{32}$P]*phosphorylcholine.* [$^{32}$P]Phosphorylcholine was prepared according to the method of Riley (1944) and purified as described by Plimmer & Burch (1937). Calcium ions were removed by passing the solution

through an IR-120 ($K^+$ cycle) column. The material prepared in this fashion had a specific activity of 25–45 $\times 10^3$ counts/min./$\mu$mole.

Phosphorus was estimated by the method of Allen (1940).

Esterified fatty acids were estimated by the method of Stern & Shapiro (1953).

Unesterified fatty acids were determined as described by Entenman (1957) after purification (Fairbairn, 1945).

*Counting of radioactive samples.* All samples were plated at infinite thinness on nickel planchet either 1·6 or 2·5 cm. in diam. The planchets were counted in a Tracerlab SC16 windowless counter with a conventional scaler. When both $^{32}P$ and $^{14}C$ were present simultaneously, these were differentially counted by interposing a thin aluminium disk, which effectively absorbed all the $^{14}C$ counts. The $^{32}P$ counts were corrected for absorption in the aluminium disk.

*Calculations.* The results given in the tables for experiments in liver slices always refer to the total counts present in 400 mg. of slices. The presence of variable quantities of fat mobilized from the depots in the livers of the treated animals makes this figure more useful than the more usual 'specific activities'.

## RESULTS

*Incorporation of [1-$^{14}$C]acetate and [1-$^{14}$C]palmitate into liver neutral lipids and phospholipids.* The effect of pituitary growth hormone on the rate of incorporation of [1-$^{14}$C]acetate into the neutral lipids and phospholipids of rat-liver slices is shown in Table 1. In all six experiments shown in Table 1, the rate of incorporation of the precursor into phospholipids is decreased, the factor varying between three- and ten-fold. An inhibition of the rate of incorporation into the neutral lipids of the same slices is also observed although this is not so great as that observed for the phospholipids.

Table 2 shows similar experiments in which the rates of incorporation of [1-$^{14}$C]acetate and [1-$^{14}$C]-palmitate into neutral lipids and phospholipids are compared with slices from the same liver for both substrates. It will be seen that the hormone also inhibits the incorporation of [1-$^{14}$C]palmitate into the lipid fractions although it should be noted that the effect on the incorporation of the labelled palmitate is less than that observed with acetate.

*Activation of palmitic acid.* Since palmitic acid has to be activated before it enters the pathway of phospholipid or neutral-lipid synthesis, the effect of growth hormone on the rate of the activation process was measured. The results of these experiments are shown in Table 3. It is apparent that there is no difference in the rate at which the

Table 1. *Effect of pituitary growth hormone on the incorporation of acetate* [1-$^{14}$C]*into the 'neutral fats' and phospholipids of rat-liver slices*

Each flask contained 400 mg. of slices in 5 ml. of Krebs–Ringer bicarbonate. Sodium [1-$^{14}$C]acetate was added to a final concentration of 0·1 $\mu$c/ml. Gas phase, $CO_2 + O_2$ (5:95). Temp. 37°. Time 3 hr.

| | Phospholipids (counts*/100 sec./flask) | | | Neutral fats (counts*/100 sec./flask) | | |
|---|---|---|---|---|---|---|
| Expt. | Control | Hormone-treated | Treated / Control | Control | Hormone-treated | Treated / Control |
| 1 | 3 600 | 912 | 0·25 | 6 178 | 5 061 | 0·82 |
| 2 | 7 338 | 2 748 | 0·37 | 15 988 | 10 710 | 0·67 |
| 3 | 7 716 | 2 148 | 0·28 | 7 492 | 5 445 | 0·73 |
| 4 | 4 608 | 1 524 | 0·33 | 7 944 | 5 639 | 0·71 |
| 5 | 8 435 | 1 765 | 0·21 | 15 472 | 4 420 | 0·29 |
| 6 | 7 644 | 849 | 0·11 | 15 200 | 3 193 | 0·21 |

* Total counts present in 400 mg. of slices.

Table 2. *Effect of pituitary growth hormone on the incorporation of* [1-$^{14}$C]*acetate and* [1-$^{14}$C]*palmitate into phospholipids and 'neutral fats' of rat-liver slices*

Each flask contained 400 mg. of slices in 5 ml. of Krebs–Ringer bicarbonate. [1-$^{14}$C]Acetate or [1-$^{14}$C]palmitate was added to a final concentration of 0·1 $\mu$c/ml. Gas phase, $CO_2 + O_2$ (5:95). Temp. 37°. Time 3 hr.

| | | Phospholipids (counts*/100 sec./flask) | | | Neutral fats (counts*/100 sec./flask) | | |
|---|---|---|---|---|---|---|---|
| Expt. | Precursor | Control | Hormone-treated | Treated / Control | Control | Hormone-treated | Treated / Control |
| 1 | [1-$^{14}$C]Acetate | 19 720 | 2 683 | 0·14 | 69 520 | 38 083 | 0·55 |
| | [1-$^{14}$C]Palmitate | 9 360 | 3 654 | 0·39 | 85 420 | 29 250 | 0·34 |
| 2 | [1-$^{14}$C]Acetate | 12 800 | 3 728 | 0·29 | 58 630 | 47 490 | 0·81 |
| | [1-$^{14}$C]Palmitate | 11 784 | 5 470 | 0·46 | 79 520 | 56 460 | 0·71 |

* Total counts present in 400 mg. of slices.

Table 3. *Effect of pituitary growth hormone on the formation of palmitoyl-coenzyme A*

Each flask contained 7·5 m-moles of hydroxylamine hydrochloride adjusted to pH 7·4, 154 $\mu$moles of NaCl, 0·025 $\mu$mole of sodium [1-$^{14}$C]palmitate ($1{\cdot}23 \times 10^6$ counts/min./$\mu$mole) and rat-liver homogenate equivalent to 0·2 g. of liver, in a total volume of 5 ml. Gas phase, air. Temp. 37°. Time 2 hr.

| | Radioactivity of ferric hydroxamate complex (counts/100 sec./flask) | | |
|---|---|---|---|
| Expt. | Control | Hormone-treated | Treated / Control |
| 1 | 13 690 | 13 070 | 0·95 |
| 2 | 13 006 | 9 750 | 0·75 |
| 3 | 10 840 | 10 470 | 0·93 |

Table 4. *Effect of pituitary growth hormone on the incorporation of* [$^{32}$P]*orthophosphate into the phosphatidic acids of rat-liver mitochondria*

Each flask contained 250 $\mu$moles of sucrose, 15 $\mu$moles of $MgCl_2$, 3 $\mu$moles of ADP, 50 $\mu$moles of 2-amino-2-hydroxymethylpropane-1:3-diol buffer, pH 7·4, 100 $\mu$moles of sodium succinate, 0·3 $\mu$c of [$^{32}$P]orthophosphate and mitochondria from 0·7 g. of liver. Final volume 3 ml. Gas phase, air. Temp. 37°. Time 1 hr.

| | Radioactivity of the phosphatidic acids (counts/100 sec./flask) | | |
|---|---|---|---|
| Expt. | Control | Hormone-treated | Treated / Control |
| 1 | 823 | 939 | 1·1 |
| 2 | 1080 | 960 | 0·9 |
| 3 | 941 | 997 | 1·1 |

Table 5. *Effect of pituitary growth hormone on the dephosphorylation of the phosphatidic acids*

Each flask contained 200 $\mu$moles of maleate buffer, pH 6·4, 14 $\mu$moles of phosphatidic acid and rat-liver homogenate equivalent to 0·2 g. of liver in a final volume of 5 ml. Temp. 37°. Time 1 hr. Figures shown are corrected for blank values obtained in flasks incubated without substrate.

| | Inorganic phosphate released ($\mu$moles/flask) | | |
|---|---|---|---|
| Expt. | Control | Hormone-treated | Treated / Control |
| 1 | 2·74 | 3·26 | 1·2 |
| 2 | 2·30 | 2·11 | 0·9 |
| 3 | 2·46 | 2·75 | 1·1 |
| 4 | 1·91 | 2·11 | 1·1 |
| 5 | 2·24 | 2·28 | 1·0 |

labelled palmitate was incorporated into the ferric hydroxamate complex in hormone-treated animals and in controls. It is evident therefore that the lowered rate of palmitate incorporation into phospholipid in growth-hormone-treated rats cannot be accounted for in terms of a decreased rate of entry into the biosynthetic pathway.

*Incorporation of* [$^{32}$P]*orthophosphate into phosphatides.* The rate of incorporation of phosphate into phosphatides was measured by using an ATP-generating system in the presence of ADP and inorganic [$^{32}$P]phosphate. The $^{32}$P-labelled ATP so formed was used to phosphorylate glycerol and hence the phosphatides (Kennedy, 1953). The results of these experiments are shown in Table 4. Once again it will be observed that there is no difference between the experimental and control animals under the conditions used.

*Rate of conversion of phosphatidic acids into diglycerides.* The next step in the pathway of biosynthesis of phospholipids investigated was that in which the phosphatidic acids are dephosphorylated to give the diglycerides, the immediate precursors of both phospholipids and neutral triglycerides. The results of these measurements are shown in Table 5. Again, this step appears to be unaffected by previous treatment of the rat with growth hormone since in both groups of animals the rate of release of inorganic phosphate was virtually identical.

*Incorporation of* [$^{32}$P]*phosphorylcholine into phospholipids.* The last step investigated in phospholipid biosynthesis concerned the rate of incorporation of phosphorylcholine. A comparison of the rates of incorporation of [$^{32}$P]phosphorylcholine into the phospholipids of normal and growth-hormone-treated animals is shown in Table 6. Although the rate of incorporation of the phosphorylcholine increases more or less in proportion to the level of substrate present, the hormone-treated animals consistently incorporate the phosphorylcholine faster and, indeed, over the range used, the ratio treated : control remained reasonably constant at about 1·4.

Table 6. *Effect of pituitary growth hormone on the incorporation of* [$^{32}$P]*phosphorylcholine into liver phospholipid*

Each flask contained 100 $\mu$moles of phosphate buffer, pH 7·4, 20 $\mu$moles of $MgCl_2$, 1·5 $\mu$moles of cytidine triphosphate, 10 $\mu$moles of ATP, [$^{32}$P]phosphorylcholine (33 700 counts/min./$\mu$mole) as indicated above and particles derived from 0·4 g. of liver. Further additions of 1·5 $\mu$moles of ATP and 0·03 $\mu$mole of cytidine triphosphate were made every 12 min. during the course of the experiment. Final volume 3 ml. Temp. 37°. Time 1 hr.

| | [$^{32}$P]Phosphorylcholine incorporated ($\mu$m-moles/flask) | | |
|---|---|---|---|
| [$^{32}$P]Phosphorylcholine added ($\mu$moles/flask) | Control | Hormone-treated | Treated / Control |
| 4 | 4·6 | 6·3 | 1·36 |
| 10 | 6·9 | 9·8 | 1·42 |
| 18 | 9·5 | 12·3 | 1·29 |
| 20 | 14·1 | 21·6 | 1·53 |

*Effect of growth hormone on the level of the lipids in the liver and the rates of incorporation of* [1-$^{14}$C]*acetate and* [$^{32}$P]*orthophosphate into these lipids.* The results shown in the foregoing tables only partially account for the degree of inhibition of acetate incorporation into phospholipids and they direct attention to the possibility that the sevenfold reduction found may have been due, in part, to a dilution of the labelled precursors by one or more of the lipids mobilized to the liver under the influence of growth hormone (Szego & White, 1949; Greenbaum & McLean, 1953*b*). In order to evaluate the degree of dilution the levels of the di- and triglycerides, phospholipids, cholesterol and cholesterol esters have been measured in the livers of normal and growth-hormone-treated rats and the incorporation of [1-$^{14}$C]acetate and [$^{32}$P]orthophosphate into these fractions has been determined. The results are shown in Table 7. The level of free fatty acids is not included in the table since it was shown by Fairbairn (1945) that hydrolysis of phospholipids to free fatty acids occurs rapidly on incubation of rat livers *in vitro*. For this reason a separate determination was made of free fatty acids in livers freshly removed from normal and hormone-treated animals. Titration of the fatty acids showed no difference between the experimental and control groups.

Table 7 shows the scale of the mobilization of triglycerides to the livers of the treated animals. Ratios as high as six have been obtained for the comparison of triglycerides between hormone-treated and control rats. It also shows that, amongst the other lipids, only diglycerides are significantly changed; the increase, by about 40%, is reproducible. Cholesterol, cholesterol esters and phospholipids remain relatively unchanged in amount.

The most outstanding difference to be observed in the values obtained in the incorporation studies is the sixfold decrease in the specific activity of the triglyceride fraction (Table 7, columns 4 and 5). This very large reduction may be accounted for by the combined action of two factors. First there is a definite decrease in the total amount of triglyceride synthesized in the livers of the growth-hormone-treated rats (columns 7 and 8), and accompanying this there is a marked mobilization of neutral fat to the livers of the treated animals (columns 1 and 2). Each of these factors lowers the specific activity about 2·5-fold. Similarly, lowered specific activity of the diglycerides in the hormone-treated rats can be accounted for in terms of a lowered rate of synthesis and a dilution; both of these factors are, however, less than with triglycerides.

With $^{32}$P as a precursor there is little difference in incorporation into either lecithins or kephalins

Table 7. *Effect of pituitary growth hormone on the levels and on the incorporation of* [1-$^{14}$C]*acetate and* [$^{32}$P]*orthophosphate into the lipids of the liver*

Results are derived from the combined livers of four rats in each group.

| | Total wt. (mg.) | | | Specific activity (counts/100 sec./mg.) | | | Total incorporation (counts/100 sec.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Hormone-treated | Treated / Control | Control | Hormone-treated | Treated / Control | Control | Hormone-treated | Treated / Control |
| Diglycerides | 70·4 | 99 | 1·4 | 1 361 | 575 | 0·42 | 95 810 | 56 900 | 0·59 |
| Triglycerides | 287 | 667 | 2·3 | 1 851 | 280 | 0·15 | 531 000 | 186 700 | 0·35 |
| Lecithins ($^{14}$C) | 425 | 510 | 1·2 | 627 | 235 | 0·37 | 266 500 | 119 800 | 0·45 |
| Lecithins ($^{32}$P) | | | | 469 | 429 | 0·91 | 199 300 | 218 800 | 1·1 |
| Kephalins ($^{14}$C) | 126 | 151 | 1·2 | 815 | 279 | 0·34 | 102 700 | 42 100 | 0·41 |
| Kephalins ($^{32}$P) | | | | 539 | 543 | 1·0 | 67 900 | 82 000 | 1·2 |
| Sterols* | 51 | 54 | 1·1 | 4 064 | 6 157 | 1·5 | 207 300 | 332 500 | 1·6 |
| Sterol esters | 32·2 | 39·4 | 1·2 | 670 | 833 | 1·2 | 21 570 | 32 840 | 1·5 |
| Pigments etc. | 14·5 | 6·8 | 0·5 | 633 | 5 376 | 8·5 | 9 190 | 36 720 | 4·0 |

* The fraction called 'sterols' in this table is composed of up to 98–99% of cholesterol (Barron & Hanahan, 1958) and has been called cholesterol in this paper.

after treatment with growth hormone. The slightly higher phospholipid content of the livers of the treated animals (column 1 and 2) results, however, in a small rise in the total counts measured in these two groups. When [1-$^{14}$C]acetate is used the specific activity of these two fractions is reduced by about 60–65 % in the hormone-treated animals and the total counts by 55–60 %.

The specific activity of the cholesterol [the fraction called the sterols in the table is composed of up to 98–99 % of cholesterol (Barron & Hanahan, 1958) and will be called cholesterol in this paper] is raised in the hormone-treated animals as are the total counts. This is in agreement with previous observations of an increased rate of cholesterol synthesis accompanying a decreased rate of fatty acid synthesis. An increase is also found, both in specific activity and in the total counts incorporated, in the sterol esters. In the group 'pigments etc.' of the table, Barron & Hanahan (1958) included hydrocarbons. On examination of the values for the specific activity of the fractions as they emerged from the column it is clear that this heterogeneous group of compounds contains some substances of extremely high specific activity (up to 16 000 counts/100 sec./mg.), and in view of the fact that squalene is eluted with hexane, as are these pigments, it would appear that these high counts may be ascribed, amongst other things, to cholesterol precursors.

## DISCUSSION

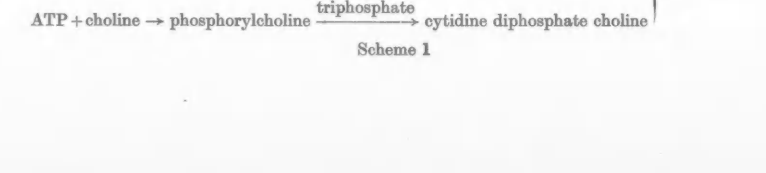
The results of the present series of experiments confirm and extend the previous results of Greenbaum & Glascock (1957) in that growth hormone is shown to cause a marked inhibition of the incorporation of acetate into phospholipids and neutral lipids. The degree of decrease of incorporation into phospholipids varies somewhat from animal to animal, but is generally about fivefold. Although the incorporation of acetate into the neutral fats does not appear to be affected to the same degree (Table 1) there is, in fact, a strong inhibition. The 'neutral-lipid' fraction is of a composite nature and includes triglycerides, diglycerides, cholesterol and cholesterol esters. When this group is fractionated to obtain pure di- and tri-glyceride fractions then it is found that growth hormone greatly decreases the incorporation of acetate into these substances also (see Table 7).

To try to find where growth hormone affects the rate of phospholipid synthesis measurements have been made of some of the component reactions of the pathway of phospholipid biosynthesis in normal and growth-hormone-treated rats. This pathway may be indicated as in Scheme 1.

The first comparisons were made of the spans acetate to phospholipid and palmitate to phospholipid (Tables 1 and 2). It was found that whereas the rate of incorporation of acetate was usually decreased fivefold (and sometimes eightfold) the decrease of the rate of incorporation of palmitate never exceeded threefold and was more usually between two- and three-fold. In this context Jedeikin & Weinhouse (1954) found that acetate incorporation into phospholipid was more affected by starvation than was the incorporation of palmitate.

This discrepancy between the decrease in rates of incorporation of acetate and palmitate could arise either from a direct effect of the hormone on the long-chain fatty acid-activating systems or on the enzymic steps which convert acetate into a long-chain acyl-CoA. A comparison was therefore made of the rate of activation of palmitate by unfortified homogenates of liver taken from normal and growth-hormone-treated animals. As will be seen from Table 3, there was an active incorporation of labelled carbon into the ferric hydroxamate complex in both sets of animals and, indeed, no difference could be detected in the rate of formation of palmitoyl-CoA in the two groups. It is thus possible to locate one of the sites of growth-hormone activity as lying on the pathway between acetate and long-chain acyl-CoA.

Another site of growth-hormone activity appears to exist in the second portion of the chain, i.e. the part leading from palmitoyl-CoA to phospholipid. In an attempt to localize this site further, comparisons were made of the steps involved, namely (*a*) the formation of phosphatidic acids as measured by the rate of incorporation of $^{32}$P via the intermediate formation of labelled ATP and α-glycerophosphate, (*b*) the dephosphorylation of phosphatidic acid to diglyceride, and (*c*) the conversion of




Scheme 1

phosphorylcholine into phospholipid. In the first of these steps it has been shown that the radioactivity of the extracted phosphatidic acids is a measure of their rate of formation (Kennedy, 1953; Marinetti, Erbland & Albrecht, 1957). Table 4 shows that the radioactivities of the phosphatidic acids extracted from the livers of normal and growth-hormone-treated rats are virtually identical and it may therefore be concluded that the esterification of α-glycerophosphate by palmitoyl-CoA is unimpaired in treated rats. This is also true of the second reaction studied, the dephosphorylation of phosphatidic acids to diglycerides (Table 5). In the third reaction measured, the incorporation of phosphorylcholine into phospholipid, a small but definite increase in the incorporation rate in treated animals was recorded, i.e. this reaction would not account for the decreased rate of palmitate incorporation which had been found in growth-hormone-treated animals.

The lack of any enzymic differences between the two groups of rats studied directed attention to the possibility that the lowered rate of palmitate incorporation into phospholipid in the livers of growth-hormone-treated rats could be due to a dilution of the label by unlabelled precursor at one of the steps shown on the Scheme. For this reason the liver lipids of normal and control rats were chromatographed on silicic acid columns and the levels of the different lipids measured (Table 7).

While almost all the fractions were present in slightly greater quantity in the hormone-treated animals, only two of the fractions, the di- and triglycerides, were significantly raised. Of these two, only the diglyceride could act to dilute the isotope incorporated into the phospholipid. The 40% increase in the level of this fraction in the livers of the hormone-treated rats could account for most of the decreased incorporation of palmitate into phospholipid, both on a basis of specific activity or as total counts incorporated. It should be noted, however, that the diglyceride fraction is again a complex mixture. It is composed of diglycerides that give rise to triglycerides but not phospholipids, diglycerides that give rise to phospholipids and not triglycerides, and diglycerides that give rise to both. The present data do not show whether all these different fractions are affected by growth hormone, or only some of them. A more detailed fractionation of this large group would help to throw some light on this point.

Thus the results point to two possible regions at which growth hormone could produce an effect which would result in the great decrease in the incorporation of acetate. The first of these is in the enzymic span acetate → palmitoyl-CoA; the second is probably a dilution of the isotopically labelled intermediates at the level of the diglycerides.

## SUMMARY

1. The incorporation of [1-$^{14}$C]acetate and [1-$^{14}$C]palmitate into liver phospholipids has been studied in normal and growth-hormone-treated rats. The hormone treatment greatly reduces the rate of incorporation of these substances, acetate more so than palmitate.

2. Measurements have been made of (*a*) the rate of the activation of palmitate to palmitoyl-coenzyme A, (*b*) the incorporation of $^{32}$P-labelled adenosine triphosphate into phosphatidic acid, (*c*) the dephosphorylation of phosphatidic acid and (*d*) the incorporation of phosphorylcholine into phospholipid in normal and treated animals. The first three reactions proceeded at essentially the same rate in both groups of rats whereas the fourth reaction, the incorporation of phosphorylcholine, proceeded faster in the treated animals.

3. Measurements have been made of the levels of the various lipid fractions in the livers of normal and treated rats and the incorporation of [1-$^{14}$C]-acetate into these fractions. There is a significant increase in the levels of the di- and tri-glycerides.

4. It is suggested that the reduced rate of incorporation of acetate into phospholipids is caused partly by a slowing of the enzymic span acetate → palmitoyl-coenzyme A, and partly by an increased dilution of the labelled diglyceride by the greater amounts of unlabelled diglyceride present in the livers of the treated rats.

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# The Demethylation of Griseofulvin by Fungi

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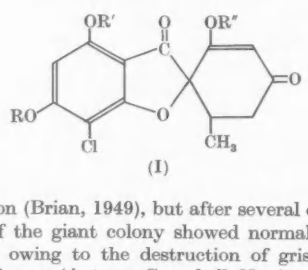
As one method of obtaining strains of *Microsporum canis* resistant to griseofulvin [7-chloro-4:6:2′-trimethoxy-6′-methylgris-2′-en-3:4′-dione (I: R, R′, R″, Me)] the organism was grown on agar containing griseofulvin at a low concentration. At first the hyphae exhibited typical curling and




(I)

distortion (Brian, 1949), but after several days the edges of the giant colony showed normal hyphal growth, owing to the destruction of griseofulvin by the fungus (Aytoun, Campbell, Napier & Seiler, 1960). Biological assay showed that griseofulvin had disappeared from the substrate, and paper chromatography detected the presence of another substance, a new compound that has been characterized as 7-chloro-4-hydroxy-6:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (I: R, R″, Me; R′, H) (4-demethylgriseofulvin).

In continuation of this work, many other fungi have been tested for their ability to metabolize griseofulvin. Some rapidly destroyed the griseofulvin; from two of them other griseofulvin metabolites were recovered and characterized. *Botrytis allii* and *Cercospora melonis* produced griseofulvic acid (I: R, R′, Me; R″, H) (Grove, MacMillan Mulholland & Rogers, 1952), and 6-demethylgriseofulvin (I: R, H; R′, R″, Me) (Barnes & Boothroyd, 1961) respectively.

## EXPERIMENTAL

*Paper chromatography.* The solvent system and methods used have been reported by Barnes & Boothroyd (1961). All the reported compounds gave blue fluorescent spots when paper chromatograms of these compounds were examined under ultraviolet light; $R_F$ values are: griseofulvin, 0·90; 4-demethylgriseofulvin, 0·70; griseofulvic acid, 0·20; 6-demethylgriseofulvin, 0·15.

*Biological assay.* The griseofulvin content of the various substrates was determined by a serial dilution method with *Alternaria solani* as test organism. Griseofulvin solutions absorbed on filter paper were allowed to diffuse through a cellophan disk placed on the damp filter paper so as to come in contact with germinating *A. solani* spores. End-point concentrations caused the hyphae to exhibit regular curling without stunting.

*Spectrophotometry.* Ultraviolet spectra were determined in ethanol containing 1% (v/v) of either 2N-HCl or aq. 2N-$NH_3$ soln. Infrared spectra were determined in $CHBr_3$ unless otherwise stated, in a Perkin–Elmer model 21 instrument with a NaCl prism.

*Materials.* The [$^{36}$Cl]griseofulvin was obtained by fermentation as described in a previous paper (Barnes & Boothroyd, 1961), in which are also described methods of determining the amount of radioactive material. The [$^{36}$Cl]griseofulvin used had a specific activity of 1·48 $\mu$c/m-mole.

*Organisms.* The following organisms were used: *Alternaria solani*, Boyce-Thompson Institute; *Botrytis allii*, C.M.I. Culture no. 42078; *Cercospora melonis*, Cheshunt Experimental Station; *Microsporum canis*, isolated from human ringworm by Dr J. Gentles, University of Glasgow.

*Methods of incubation.* With a spore suspension prepared from Sabouraud's malt–agar slopes as inoculum, cultures of the various organisms were prepared in Glaxo mould-culture flasks containing Sabouraud's malt broth (200 ml.). This was prepared by dissolving maltose (40 g.), Evans peptone (10 g.) and Oxoid malt extract (24 g.) in distilled water (1 l.) and adjusting to pH 7. Slopes were prepared from this broth by adding agar (20 g.). After incubation at 27° for several days, the exact time depending upon the organism used, a thick-growing felt of mycelium covered the surface of the broth. The broth was then poured off; after the felts had been washed twice with sterile water, a sterile suspension of griseofulvin (200 ml.) was added to each flask. Two concentrations of griseofulvin suspension were used as a routine, 40 and 100 $\mu$g./ml. They were prepared by dissolving griseofulvin in a small volume of acetone and pouring this solution into a large volume of water. The 40 $\mu$g./ml. suspension contained a high proportion of the griseofulvin in solution (approx. 25 $\mu$g./ml.) and was used in initial tests for metabolism; the 100 $\mu$g./ml. suspensions were used for preparing the pure metabolites.

The griseofulvin suspensions were incubated with the different felts for several days, and breakdown of griseofulvin was followed by bioassay and paper chromatography in order to determine the optimum harvest time.

## ISOLATION AND IDENTIFICATION OF METABOLITES

*4-Demethylgriseofulvin. Microsporum canis* felts were grown on Sabouraud's malt broth for 9 days before adding the griseofulvin substrate; incubation was then continued for 7 days before harvesting. At harvest the aqueous layer was decanted from the felts and clarified by filtration before extraction with $CHCl_3$ ($2 \times 0{\cdot}33$ vol.). Complete recovery by weight was obtained from 40 $\mu$g./ml. suspensions of griseofulvin, but only 60% when 100 $\mu$g./ml. suspensions were used. In these experiments the larger particles of griseofulvin were unchanged and were removed during the clarification.

The $CHCl_3$ extracts from several large-scale incubations were combined and evaporated to dryness, leaving a brown solid (0·27 g.). This material was purified by chromatography on acid alumina (15 g., Woelm) that had been deactivated to Brockmann grade 4 by treatment with water. The crude solid dissolved in benzene (5 ml.) was poured on the alumina column, prepared from a benzene slurry, and eluted with the same solvent (50 ml.). Griseofulvin (5 mg.) was eluted in this fraction. The eluting solvent was then changed to 2% of ethanol in benzene, and more material (162 mg.) was eluted. Further elution with higher concentrations of ethanol and finally pure ethanol gave only small amounts of amorphous solids, which could not be purified. The solid from the main fraction was first crystallized by adding water to a hot solution of the material in 50% acetic acid and allowing this to cool. After several recrystallizations from a mixture of ethyl acetate and light petroleum (b.p. 60–80°) and finally of methanol and water, a constant m.p. 113–115° (uncorr.) was obtained. The melting point varied greatly with rate of heating; on rapid heating considerably higher values were observed. The figure quoted is that obtained by slow heating on a microscope hot-plate, taking the first breakdown of crystals to a viscous liquid as the melting point (Found: C, 54·3; H, 5·0; Cl, 10·1; OMe, 17·6. $C_{16}H_{15}O_6Cl,H_2O$ requires C, 53·9; H, 4·8; Cl, 10·0; OMe, 17·4%); light-absorption max. for the hydrated crystals in acid soln., 236 and 290 m$\mu$ (log $\epsilon$ 4·39 and 4·42 respectively); in alkaline soln., 246, 290 and 368 m$\mu$ (log $\epsilon$ 4·42, 4·35 and 3·88 respectively). The pure material gave a green–brown colour with ethanolic $FeCl_3$. Paper chromatograms of the material showed only one blue fluorescent spot corresponding to the one developing in the griseofulvin substrate on incubation with *Microsporum canis*.

The metabolite (5 mg.) was treated with a solution of diazomethane in ether. After 2 hr. the ether and excess of diazomethane were evaporated, and the residual solid was crystallized from ethanol. These crystals showed no depression of melting point when mixed with an authentic sample of griseofulvin. They also had the same ultraviolet-absorption spectrum and the same $R_F$ on paper chromatography as griseofulvin.

*Structure of 4-demethylgriseofulvin.* Microanalysis, together with the evidence that diazomethane converted this material into griseofulvin, showed that demethylation of one of the three methoxy groups had taken place during its formation. Griseofulvic acid (I: R, R′, Me; R″, H) (Grove *et al.* 1952) and 6-demethylgriseofulvin (I: R, H; R′, R″, Me) (Barnes & Boothroyd, 1961) both had different melting points and ultraviolet and infrared spectra, so that 4-demethylgriseofulvin (I: R, R″, Me; R′, H) is the only possible structure. Corroboration was obtained from infrared spectra in which the peak associated with the carbonyl group in the five-membered ring moved from 1705 cm.$^{-1}$ in the griseofulvin spectrum to 1690 cm.$^{-1}$ in that of the metabolite. This change of frequency is consistent with the presence of a bonded ketone in the new compound. Duncanson, Grove, MacMillan & Mulholland (1957), using related compounds, report a similar change of frequency (18 cm.$^{-1}$) and also discuss changes in the ultraviolet spectra of these compounds caused by different pH values of the solvent. Reference to their work led to the structure 6-demethylgriseofulvin for the griseofulvin metabolite excreted in the urine (Barnes &

Boothroyd, 1961), since major changes in ultraviolet spectra between acidic and alkaline solution can be predicted for the *p*-hydroxy ketone structure present in this compound. Similarly, little change in the main *K* band would be expected for the *o*-hydroxy ketone structure present in this new griseofulvin metabolite, 4-demethylgriseofulvin.

*Griseofulvic acid. B. allii* felts were grown on Sabouraud's malt broth for 4 days before adding the griseofulvin substrate; incubation was then continued for 6 days before harvest: [$^{36}$Cl]griseofulvin was used in this experiment.

The solution (5 l., pH 6·3), decanted from the flasks at harvest, was acidified to pH 2·5 and extracted with $CHCl_3$ (3 × 1·6 l.). The combined $CHCl_3$ extracts were concentrated to 100 ml. and extracted with 2 N-NaOH (3 × 25 ml.). Evaporation of the $CHCl_3$ phase produced a brown oil (40 mg.), which was shown by paper chromatography to contain griseofulvin. The combined alkaline extracts were acidified and extracted with $CHCl_3$, and the $CHCl_3$ solution was evaporated to a brown solid (208 mg.). This was extracted with benzene, and the residue, a colourless solid (100 mg.), was crystallized from aqueous methanol to yield colourless needles, m.p. 254–256° (decomp.) (uncorr.); $[\alpha]_D^{20} + 384°$ ($c$ = 0·98 as sodium salt in aqueous methanol); light-absorption max. in acid soln., 270 and 291 mμ (log. ε 4·36 and 4·34 respectively), in alkaline soln. 290 mμ (log ε 4·69). Grove *et al.* (1952) report for griseofulvic acid m.p. 255–258° (decomp.) and $[\alpha]_D^{22} + 399°$ ($c$ = 1·004 as sodium salt in aqueous methanol). A sample of griseofulvic acid prepared by their method gave identical ultraviolet and infrared spectra (Nujol mull) and had the same $R_F$ (0·2) on paper chromatography as the crystalline material from *B. allii* incubation.

Preliminary work on the distribution of griseofulvin and its metabolites after incubation with *B. allii* felts showed that about 50 % of the radioactive material used was present in the decanted solution; the remainder extracted from the felts with acetone was shown to be unchanged griseofulvin. To check that no other major metabolites were present in the $CHCl_3$ extract, the benzene wash of the original solid (208 mg.) was evaporated to dryness (103 mg.). This solid was then purified by countercurrent distribution with benzene–methanol–0·002 N-HCl (2:2:1, by vol.) as solvent system. After 50 transfers the distribution of radioactive material was determined by counting samples from each tube. Only one peak of radioactivity, in tubes 12–27, was observed; this contained 90 % of the input and from it only griseofulvic acid was isolated.

Griseofulvin incubated for 6 days with Sabouraud's medium alone was shown to be unchanged.

*6-Demethylgriseofulvin. C. melonis* felts were grown on Sabouraud's malt broth for 6 days before adding griseofulvin substrate; incubation was then continued for 2–3 weeks before harvest.

The solution (7·4 l.) decanted from the felts was adjusted to pH 2 and extracted with $CHCl_3$ (4 × 2 l.). The dried $CHCl_3$ extract was evaporated to a brown gum (242 mg.), which gave two blue fluorescent spots ($R_F$ 0·15 and 0·90) on paper chromatography. The solid was dissolved in ethyl acetate (60 ml.) and extracted with N-NaOH (2 × 20 ml.); this alkaline solution was acidified and extracted with $CHCl_3$; on evaporation to dryness the extract gave a brown gum (82 mg.), which was purified by chromatography on Whatman 3 MM paper with the usual solvent system, although running time was increased to 24 hr. The slow-running constituent appeared as a fluorescent band, which was detected under ultraviolet light. These strips of paper were eluted with acetone for 24 hr. Evaporation of the acetone left a colourless gum (52 mg.), which was purified by crystallization from benzene to give fine colourless needles (8 mg.) m.p. 276–278°, showing no depression of melting point with an authentic sample of 6-demethylgriseofulvin. Infrared and ultraviolet spectra were also identical with those of the authentic sample.

## DISCUSSION

*Metabolism of griseofulvin by fungi.* During our work it has been demonstrated that the enzymic breakdown of griseofulvin by fungi can give rise to at least three different products, all of them monodemethylgriseofulvins. The amounts of these materials did not account for all the griseofulvin metabolized, but we could not find any other breakdown products among the metabolites of the three organisms used. These demethylated compounds could either be end products of the breakdown of griseofulvin or possibly the first stage of a more extensive degradation to less easily recognized materials. A similar hypothesis to the latter has already been proposed by Wright & Grove (1957) for the decomposition of griseofulvin by a *Pseudomonas* sp. In this case, prolonged incubation gave no recognizable products except chloride ions, suggesting rupture of the aromatic ring, but shorter incubation gave a product thought to be griseofulvic acid.

Wright & Grove (1957) also found that griseofulvin was destroyed by a fungus belonging to the Dematiaceae and present in the soil, but they characterized no breakdown products. Abbot & Grove (1959) reported the slow breakdown of griseofulvin by the two fungi, *B. allii* and *Mucor ramannianus*. Thorough investigation of the

products resulting from the incubation of griseofulvin with *Mucor ramannianus* yielded no recognizable material, although an unidentified phenolic substance, m.p. 142–145°, was isolated from the culture filtrate. From the evidence available, this phenolic substance does not appear to be one of the metabolic products discussed here.

## SUMMARY

1. Incubation of griseofulvin with fungi gave rise to various demethylated metabolites. Thus, 7-chloro-4-hydroxy-6:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (4-demethylgriseofulvin) was produced by *Microsporum canis*, griseofulvic acid (2′-demethylgriseofulvin) by *Botrytis allii* and 7-chloro-6-hydroxy-4:2′-dimethoxy-6′-methylgris-2′-en-3:4′-dione (6-demethylgriseofulvin) by *Cercospora melonis*.

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# The Effect of Glucose and Acetate on Milk Secretion by the Perfused Goat Udder

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Acetate is now generally accepted as the major energy source for most ruminant tissues. A great deal of evidence, summarized by Folley (1956), suggests that acetate is more important than glucose in the metabolism of the ruminant mammary glands. Not only is acetate directly incorporated into milk fat, but the oxygen consumption of ruminant mammary slices taken during lactation is higher with acetate as substrate than with glucose (Folley & French, 1950). However, recent work in conscious lactating goats (Linzell, 1960) shows that the udder removes on average 10·3 g. of glucose, 3·2 g. of acetic acid, and at least 650 mg. of α-amino acid nitrogen from the blood passing through it for every 100 ml. of milk produced. Compared with the uptake in non-lactating glands, these figures represent a threefold increase in the rate of uptake for acetate but a tenfold increase for glucose. Of the glucose taken up by the lactating gland only 50 % can be accounted for as lactose. These considerations suggest an important metabolic function for glucose, apart from its confirmed role in the formation of lactose.

We therefore felt that the influence of acetate and glucose upon mammary metabolism should be re-examined. It has not been possible to do this satisfactorily either in the whole animal or with tissue slices. In the whole animal, glucose or acetate cannot be eliminated entirely, and, in the tissue slice, it is not easy to measure the amount and composition of milk secreted. These are both possible in an isolated perfused mammary gland and we have used the perfused goat's udder for this purpose. In our experiments the glands are removed under spinal anaesthesia with an anoxic period of only a few minutes and perfused with a simplified perfusate of known composition. With this preparation we have shown that for the first 2 hr. milk is formed from endogenous substrates, but after that it is only produced if a substrate mixture is run into the perfusate continuously at a rate appropriate to the amount of milk being formed. The mixture used contains glucose, acetate, amino acids and minerals in the proportions that are removed from the blood by the lactating goat's udder *in vivo*. In this paper we report the effects of varying the proportion of acetate and glucose in this mixture, leaving the other components constant.

## METHODS

The method of perfusion (Hardwick & Linzell, 1960) included the modifications mentioned to maintain a high blood flow throughout perfusion and the incorporation of

an artificial kidney. The glands were removed aseptically under spinal (epidural) anaesthesia. The perfusate consisted of washed goat red cells suspended in a modified Krebs bicarbonate solution [composition (g./l.): NaCl, 7·70; $NaHCO_3$, 2·10; KCl, 0·230; $CaCl_2$ (anhydrous) 0·282; $MgSO_4,7H_2O$, 0·294; $KH_2PO_4$, 0·162] containing instead of albumin the total proteins of defibrinogenated plasma (70 g./l.). The substrates required for milk secretion were run into the perfusate continuously throughout the perfusion by a motor-driven syringe.

The protein for the perfusate was prepared by adding ether (11% of the plasma volume) to plasma cooled to 0° (Kekwick & Mackay, 1954). The precipitate was removed, and the supernatant dialysed for three 24-hr. periods against 10 vol. of NaCl solution (2–3 g./l.) and concentrated by freeze-drying. The NaCl content was reduced by further dialysis against distilled water and measured by flame photometry before adding the necessary Krebs salts.

The artificial kidney was inserted between a reservoir and the subsidiary circulating pump, and consisted of 10 m. of dialysis tubing 6 mm. in diameter (Visking $\frac{8}{32}$ in.) in 20 l. of Krebs solution to which were added (/l.): glucose, 0·5 g.; acetic acid (neutralized), 0·05 g.; L-asparagine, 3 mg.; L-glutamine, 50 mg.; L-cysteine, 35 mg.; L-phenylalanine, 25 mg.; L-tryptophan, 20 mg.; L-tyrosine, 17 mg.; streptomycin and penicillin, 100 000 units; casein hydrolysate to give a total α-amino N value of 50 mg./l. Glucose or acetate was omitted from the 'kidney' when not given in the substrate solution.

The casein hydrolysate was made by refluxing 100 g. of casein in 25 ml. of formic acid (90%; density 1·2), 60 ml. of conc. HCl and 500 ml. of water for 3 days. As much acid as possible was removed by evaporating the resulting material under reduced pressure to a small volume, adding 200–300 ml. of water and re-evaporating. This evaporation was repeated three times. Activated carbon (20 g.) (Norite NK; Hopkin and Williams Ltd.) was added, the solution filtered and neutralized, and the α-amino N content checked.

The substrate solution used consisted of the mixture of amino acids and phosphate used by Hardwick & Linzell (1960). The total concentration of glucose plus acetic acid (neutralized with NaOH) was 100 g./l., but the proportions were varied in different experiments. This substrate solution was run into the perfusate continuously at a rate determined hourly by the milk yield of the previous hour. A solution of $CaCl_2$ (0·23 g./100 ml.) and $MgCl_2,6H_2O$ (67 mg./100 ml.) in water was also added at a rate of 8 ml./hr.

Milk and perfusate were analysed as described by Hardwick & Linzell (1960). Arterio-venous differences of $O_2$ and $CO_2$ were measured by taking arterial and venous samples simultaneously and analysing for $O_2$ and $CO_2$ by the standard manometric procedure of Van Slyke (Peters & Van Slyke, 1932). Consumption of $O_2$ and R.Q. were calculated from these figures and the blood flow at the time. Ketone bodies were measured in some experiments by the methods of Bakker & White (1957) and Werk, McPherson, Hamrick, Myers & Engel (1955). Glucose was sometimes checked with the glucose-oxidase method of Huggett & Nixon (1957).

Milk yields during perfusion were compared with the average hourly yield of the gland on the goat during the previous week and this is referred to as the yield before the experiment. The composition of the total milk secreted for the 3 days before the experiment was used for comparison and is referred to as 'control level'.

## RESULTS

Hardwick & Linzell (1960) have shown that the milk yield in the isolated udder is dependent on blood flow and the quantity of substrates provided. In the present experiments blood flow, after the initial phase, was 35–65 ml./100 g./min., which is in the normal range ($47 \pm 9$) for conscious lactating goats. Also, more substrate mixture was given than would have been necessary *in vivo* to make the amount of milk obtained during the perfusion. In addition, nutrients diffused into the perfusate from the artificial kidney when concentrations in the perfusate fell below those in the 'kidney', and vice versa. We therefore consider that neither low blood flow nor restriction of substrates was a factor in these experiments.

Eight glands from four goats were perfused. Since the variation in milk yield between the two glands from one goat is less than that between glands of different goats the basic experimental design shown in Table 1 was used. In addition to comparing the results obtained under these conditions, one with another, it is important to compare the glucose, acetate and amino acid uptake/100 ml. of milk *in vivo* (see introduction) and in the perfused gland, as a measure of efficiency of the latter. Comparison of $O_2$ uptake and R.Q. *in vivo* and during the perfusion is also of significance (see Fig. 5).

### *Perfusions with glucose plus acetate*

In these two experiments the substrate solution run in and the 'kidney' fluid contained glucose plus acetate (3:1, w/w; 1:1 molar ratio). The results were similar to those reported by Hardwick & Linzell (1960) and are shown in Figs. 1*L* and 2*R*. We have previously found that milk secretion during the first 2 hr. is largely from endogenous

Table 1. *Experimental design*

Experimental design showing substrates, other than amino acids, Ca, Mg and phosphate, added continuously from the start of perfusion. Alterations in substrate composition were made subsequently in glands 2*L*, 3*R*, 4*L* and 4*R*. G, Glucose; A, acetate.

| | Goat ... | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Gland | Left | G+A | A | G | A and little G |
| | Right | G | G+A | A | No addition |

substrates (see also Fig. 4*R*). The average rate of secretion after this was 41 and 47% of the yield before the experiment and was still above 10% at the 12th hr. The milk composition was essentially normal. The $O_2$ uptake was equal to that in conscious goats in declining lactation and the R.Q. was higher (Fig. 5). For each 100 ml. of milk formed the two glands took up 12 g. (estimated by using final perfusate concn. and assuming equilibrium with the 'kidney' fluid) and 19 g. (based on analysis of

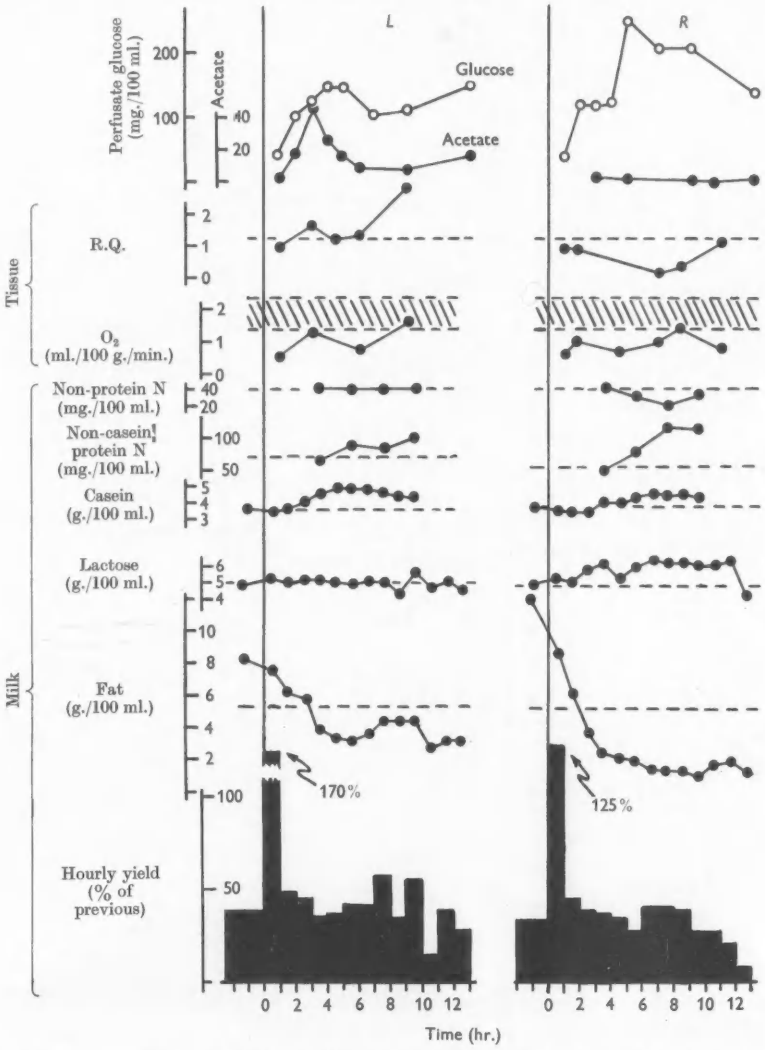


Fig. 1. Expt. no. 1. *L*, Left gland: glucose plus acetate; *R*, right gland: glucose only. The perfusion was begun at 0 hr.; points before this represent milk secreted during the removal of the glands under epidural anaesthesia. The average hourly yield for 7 days before the experiment equals 100%. Horizontal broken lines represent composition of milk produced during the 3 days before experiment; for $O_2$ and the R.Q. the broken lines indicate average values in conscious lactating goats (see Fig. 5).

perfusate and 'kidney' fluid) of glucose, 0·76 and 0·54 g. of α-amino N and 6·8 and 6·6 g. of acetate. The greater uptakes of glucose in one case and N in the other were associated with higher concentrations in the milk of lactose and N respectively.

*Perfusion with glucose only*

In both these experiments (Figs. 1*R* and 3*L*) the average rate of milk secretion after the 2nd hr. was 30 % of the yield before the experiment. The milk

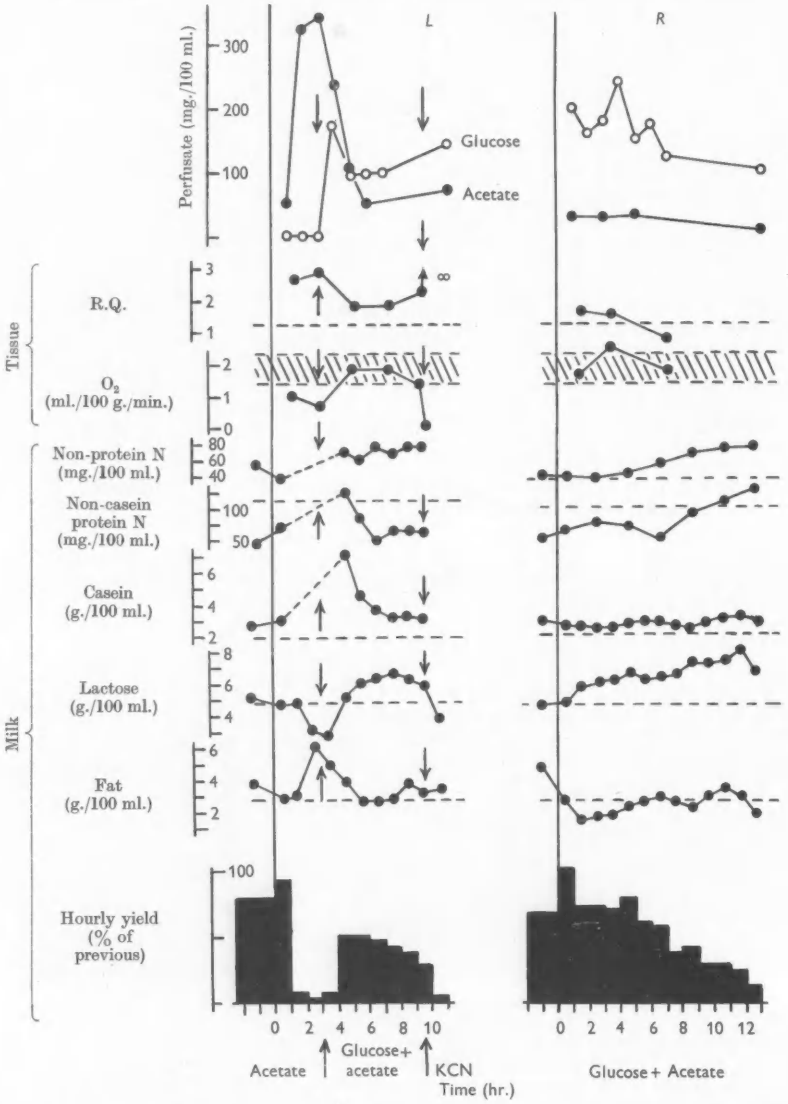


Fig. 2. Expt. no. 2. *L*, Left gland: 0–3 hr., acetate only; 3–11 hr., glucose and acetate; KCN (0·1 mM) was added at 9·3 hr. *R*, Right gland: glucose and acetate. Explanations are as in Fig. 1.

composition was in the normal range and similar to that with glucose and acetate, except that the fat concentration fell after the first 2 hr., eventually to as low as 18 and 12% of the control level. The $O_2$ consumption was significantly ($P < 0.05$) lower than with acetate and glucose and the R.Q. < 1 ($P < 0.02$) (Fig. 5). For each 100 ml. of milk formed the two glands took up 28 g. (estimated as above) and 23 g. of glucose and 0·98 and 0·66 g. of α-amino N. Again the higher

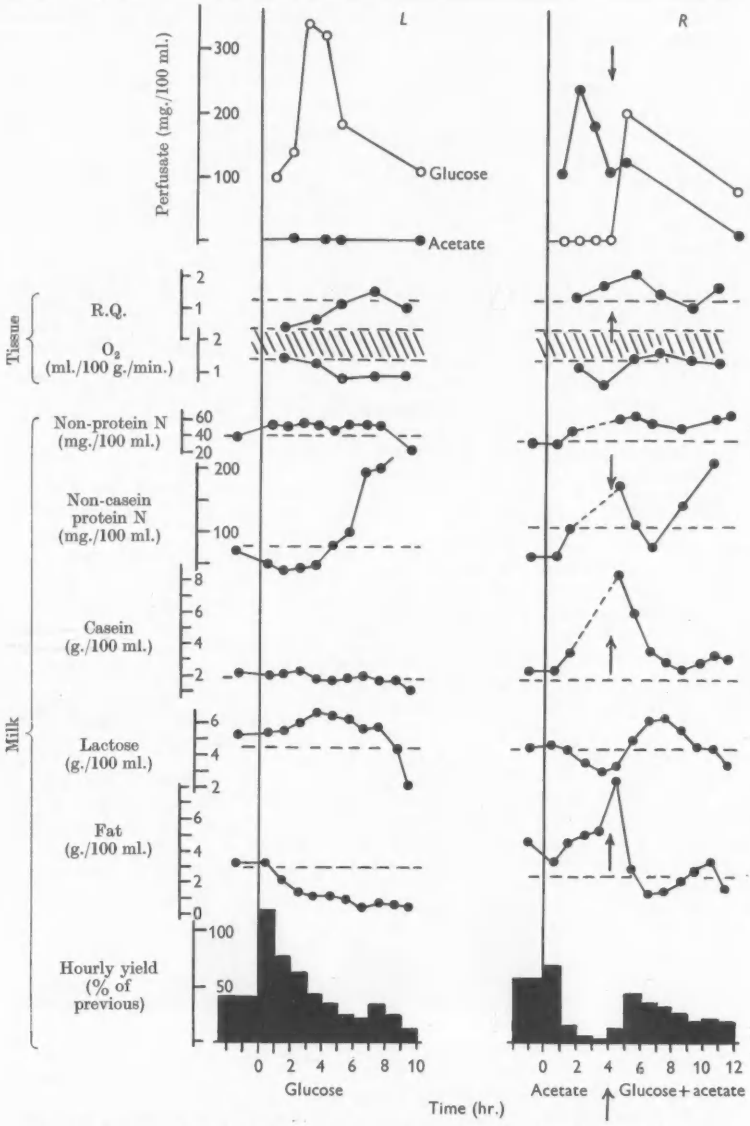


Fig. 3. Expt. no. 3. *L*, Left gland, glucose only; *R*, right gland, 0–4 hr., acetate only; 4–12 hr. glucose + acetate. Explanations are as in Fig. 1.

uptake of N corresponds with a higher total milk N.

*Perfusion with acetate only*

In these two experiments (Figs. 2*L* and 3*R*) the milk yield fell by the 3rd hr. to a very low level and was not significantly different from glands receiving no substrates (e.g. Fig. 4*R*). The samples of milk that were large enough to analyse showed a fall in lactose and a rise in casein and fat concentration. The $O_2$ uptake was similar to the value with glucose only and intermediate between those

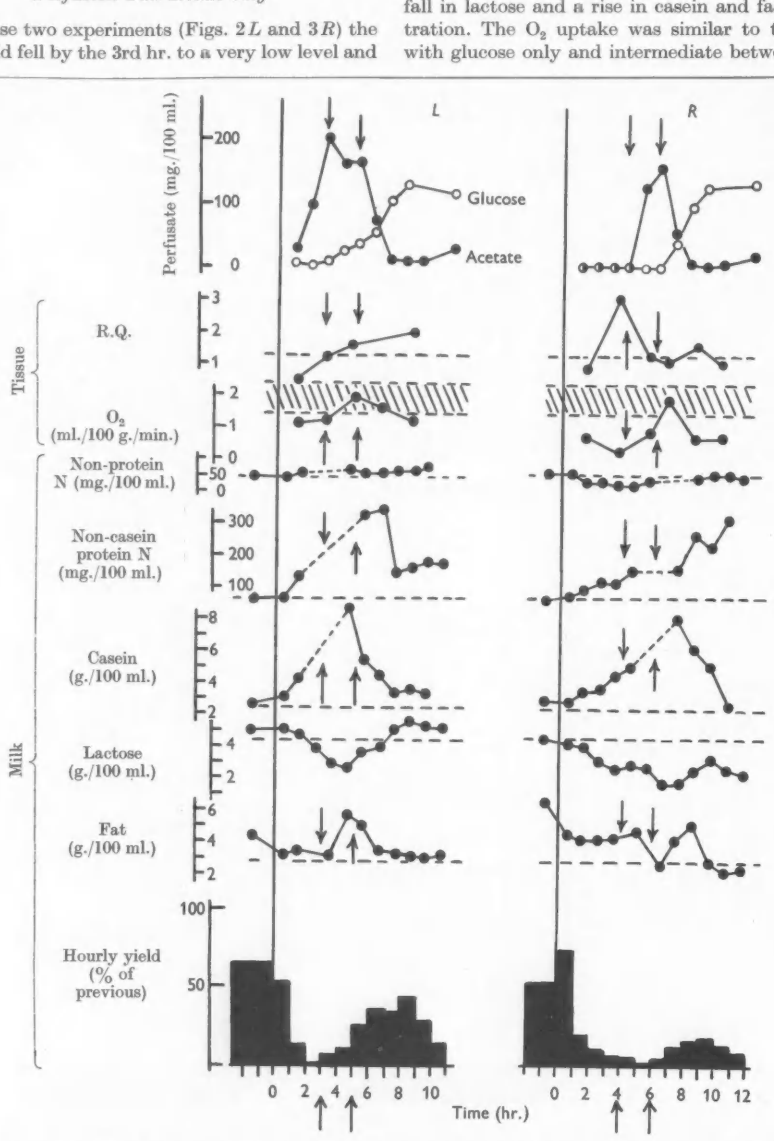


Fig. 4. Expt. no. 4. *L*, Left gland, 0–3 hr. The substrate solution contained glucose + acetate (1:27 molar ratio); 3–5 hr. (1:5 molar ratio); 5–10 hr. (1:1 molar ratio). Arrows indicate where glucose in substrate solution was increased. *R*, Right gland: 0–4 hr., no substrates; 4–6 hr., acetate + fumarate (18:1 molar ratio); 6–12 hr., glucose + acetate (1:1 molar ratio). Other explanations are as in Fig. 1.

of starved glands and those receiving glucose plus acetate. The R.Q. was markedly raised (Fig. 5). Because of the very low milk yields it seemed desirable to test the glands' ability to secrete milk. At 3 hr. in one experiment and at 4 hr. in the other, therefore, we added glucose to the 'kidney' fluid and to the perfusate to give a concentration of 50 mg./100 ml., and changed the substrate mixture being infused from one containing acetate to one containing glucose and acetate (molar ratio 1:1). There was an increase in milk yield at the next hour and thereafter it was similar to the controls receiving glucose and acetate throughout (Figs. 2*L* and 3*R*). The composition of the milk obtained after the addition of glucose showed a rise in the concentration of lactose to that seen in experiments where glucose was given throughout. In addition, initially, there was a very marked rise in the fat and casein concentration, which fell again to more normal levels as the perfusion progressed.

*Acetate plus a little glucose*

Since so little milk was produced with acetate alone an experiment was carried out (Fig. 4*L*) to see whether milk secretion was possible with a substrate solution and 'kidney' fluid containing a smaller ratio of glucose to acetate than 3:1 (w/w) (1:1 molar ratio). One gland given glucose and acetate (1:9, w/w) (1:27 molar ratio) for 3 hr. produced no more milk than the opposite gland getting no substrates. When the ratio in the substrate solution and 'kidney' fluid was increased to 3:5 (w/w) (1:5 molar ratio) for 2 hr. there was only a slight increase in yield. To test the ability of the preparation to secrete, we added glucose to the perfusate and 'kidney' fluid (50 mg./100 ml.) at 5 hr. and changed the substrate solution to glucose, amino acids and phosphate only, because at that time the acetate level of the perfusate was high. The milk yield increased during the subsequent 4 hr. to 44% of the yield before the experiment. The level of acetate in the perfusate fell to normal within 2 hr. so that we had to add it in the substrate mixture again.

*Acetate plus fumarate*

Terner (1956) has shown that fumarate stimulates fatty acid synthesis by mammary-gland slices in the presence of acetate. We therefore tested acetate plus fumarate as substrates on the gland (Fig. 4*R*) that was starved for the first 4 hr. At the end of the 4th hr. fumarate (5 mM) and acetate (1 mM) were added to the perfusate and the 'kidney' fluid, and the infusion of the basic substrate mixture plus acetate and fumarate (9:1, w/w; 18:1 molar ratio) was begun. There was no stimulation of milk secretion during the next 2 hr. At the end of the 6th hr. we added glucose (50 mg./100 ml.) to the perfusate and 'kidney' fluid (which still contained acetate and probably fumarate), and changed to a substrate solution containing (in addition to amino acids and phosphate which were always present) glucose, instead of acetate and fumarate. There was an increase in milk secretion during the subsequent 4 hr. (see Fig. 4*R*).

*Formation of ketone bodies*

Kronfeld & Kleiber (1959) have recently suggested that the udder itself is a source of ketone bodies in bovine ketosis, and we have looked for this in Expts. 3 and 4 by analysing the mammary lymph for total ketone bodies. All the values were below 15 mg. of acetone/100 ml. and there was no significant difference in the concentrations whether the glands were getting glucose or not.

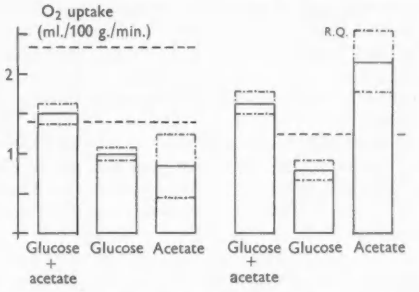


Fig. 5. Oxygen consumption and R.Q. (mean ± S.E.M.) of perfused udders with different substrates. The horizontal broken lines represent average values for conditions *in vivo* (conscious lactating goats) taken from Linzell (1960). $O_2$: upper line, goats in full lactation; lower line, in declining lactation. R.Q.: average value at all stages of lactation.

## DISCUSSION

Our results confirm the importance of acetate in the formation of milk fat, and of glucose in the formation of lactose. In addition, glucose is shown to be essential for milk secretion in our experiments.

*Role of acetate.* By the injection of labelled acetate into lactating goats, Popják, French, Hunter & Martin (1951) confirmed that acetate is the major source of milk fatty acids up to and including $C_{16}$. These acids form about 75% of milk fatty acids by weight in the goat. Our results are consistent with their findings because, in the absence of acetate, the milk fat fell to less than 20% of its previous value. This level of 20% corresponds roughly with the higher ($C_{18}$ and above) fatty acid content of normal milk. In the whole animal, apart from acetate, the glands take up enough fatty acids (free and esterified) from the

plasma to account for a major proportion of total milk fat. In our experiments only acetate was given so that the metabolism may have been abnormal. Nevertheless, fat in variable amounts was detected histologically in the secretory cells at the end of the experiment, and tests in previous perfusions have shown that neutral fat in the presence of acetate (Hardwick & Linzell, 1960) did not increase the milk yield or milk fat content, suggesting that the perfused tissue may have large stores of endogenous fat. However, the preparations then used were less active, and such experiments should be repeated under present conditions.

*Role of glucose.* The dependence of lactose synthesis on glucose was first demonstrated by Foa (1912) in the perfused sheep udder. He showed that lactose could be detected in the milk when glucose was present in the perfusate, but not when it was absent; galactose in the perfusate appeared unchanged in the milk. Kleiber *et al.* (1955) showed that 80% of milk lactose in the intact cow came from the 'plasma glucose pool'. In the conscious goat, 50% of the glucose taken up by the udder can be accounted for as lactose. We expected, therefore, that leaving out glucose from our perfusate would decrease the milk lactose, just as leaving out acetate deceased the fat concentration: this did occur. More striking than this fall, however, was the profound fall in the total milk obtained, resembling that observed when no substrates were given.

Some possible causes of the lowered rate of milk production have been eliminated. It is possible that the milk was secreted (i.e. passed into the alveolar lumina) but that the absence of glucose or the high concentrations of acetate interfered with milk ejection (i.e. prevented oxytocin from acting on the myoepithelial cells), thus allowing milk so secreted to accumulate. This is unlikely because the milk yield after glucose had been added was not greater in volume than that obtained at a similar time in experiments with glucose plus acetate given throughout, indicating that, in the absence of glucose, secretion of fluid had not been occurring. The possibility that secretion was inhibited, in the present experiments without glucose, by the high level of acetate reached in the perfusate is eliminated, since secretion started as soon as glucose was added, while the acetate level was still high. Therefore our present evidence clearly suggests that in the complete absence of glucose the secretion of milk fluid ceases, even though adequate quantities of acetate and amino acids are available.

In the absence of glucose the concentration of fat and protein rose in the small amounts of milk formed. This has also been seen in two out of six glands starved completely (Fig. 4*B*), and in one gland given adequate quantities of amino acids but very low amounts of glucose and acetate (Hardwick & Linzell, 1960; Fig. 6). The rise in the concentrations of fat and protein at the time when the lactose concentration was falling suggests that protein and fat synthesis continue so long as the specific precursors are available (they were in the present experiments and will be to a variable degree in starved glands depending on the level of endogenous substrates). If fluid secretion and lactose formation fail because there is no glucose then fat and protein, having precursors available, might be expected to accumulate in the cells until a critical concentration is reached. In this case once fluid secretion is re-established the first milk formed should have a raised concentration of fat and protein, and in fact this occurred.

Since acetate was present in high concentration and there was an oxygen uptake in excess of that in the absence of all substrates, it may be assumed that the Krebs cycle was functioning in the absence of glucose. This belief is strengthened by the failure of fumarate in the absence of glucose to have any effect on milk secretion or $O_2$ uptake (cf. Terner, 1956). Therefore it seems that the energy for secretion comes from some pathway of glucose metabolism other than the Krebs cycle and one which is not available to acetate.

One such metabolic route is the pentose phosphate pathway. Recent evidence from both ruminant (Black, Kleiber, Butterworth, Brubacher & Kaneko, 1957) and non-ruminant (McLean, 1958) mammary tissue indicates that this pathway is an important route of glucose degradation in lactating but not in non-lactating mammary tissue. McLean (1960) has suggested that the pentose phosphate pathway is linked to fat synthesis by the oxidized$\rightleftarrows$reduced triphosphopyridine nucleotide reaction and, indeed, that oxidation of glucose by this pathway might be reduced in the absence of fat synthesis needed to provide oxidized triphosphopyridine nucleotide. In our experiments casein, lactose and fluid secretion occurred in the absence of acetate when fat secretion was much reduced.

It is possible to consider milk as a liquid resembling cell fluid (i.e. high potassium and citrate, low sodium and chloride), together with the specific milk constituents lactose, casein and fat. Histochemically all the secretory cells appear to be identical, suggesting that each cell forms milk in its entirety, but it is not known to what extent the biochemical pathways controlling the formation of the various components are linked. Much recent work with isotopes shows that milk lactose, protein and fat are chiefly formed from plasma glucose, amino acids and acetate plus fat respectively. Our work with the perfused organ given variable

amounts of the three major precursors confirms this because the amount of each milk component formed is related to the amount of the precursor available. Since the formation of milk fat, lactose and protein has varied independently, this suggests that the biochemical pathways are also to a large extent independent.

*Oxygen uptake and R.Q.* These enabled us to compare the metabolism of the perfused goat gland with that of the sheep tissue slice reported by Folley & French (1950), who found only a small oxygen uptake and an R.Q. of less than unity with glucose only; with acetate only, both values were higher and with acetate plus glucose they were still further increased.

In our experiments the oxygen uptake and R.Q. of the perfused goat udder were low with glucose alone and markedly increased in the presence of glucose plus acetate, in agreement with the results on tissue slices. Our results with acetate alone differ, however, from the observations in tissue slices; we observed an oxygen uptake similar to that in the presence of glucose only, but an R.Q. greater than that in the presence of glucose plus acetate; in tissue slices both values with acetate alone were intermediate between those observed with glucose alone and with glucose plus acetate. These comparisons are probably valid, because Folley & French found similar values for the respiration of goat and sheep slices in the presence of acetate only.

## SUMMARY

1. The influence of acetate and glucose on the respiration and milk production and composition has been studied in isolated goat udders perfused with a simplified medium and receiving adequate calories, amino acids and minerals.

2. In glands receiving glucose plus acetate the oxygen uptake and respiratory quotients were similar to those of udders of conscious goats in declining lactation, and milk, of reasonably normal composition, was secreted for 12 hr. at about 50% of the level before the experiment.

3. Glands given glucose only had a lower oxygen uptake, and R.Q. $< 1$, and the milk yield was slightly reduced to about 40% of the previous level. Milk fat concentration fell to $< 20\%$ of the level observed in milk secreted before the experiment.

4. With acetate only, the oxygen uptake was equal to that with glucose but the respiratory quotients was higher than that observed with glucose plus acetate. However, milk secretion fell to very low levels and was restored by adding glucose.

5. Milk secretion was not stimulated in glands receiving mainly acetate by adding small quantities of glucose or fumarate.

6. Our results confirm that acetate and glucose are used to form milk fat and lactose respectively, but show in addition that glucose is essential for the secretion of fluid. It is suggested that the secretions of fluid, lactose, protein and fat are to some extent independent processes. The relationship of these findings to the pathways of glucose metabolism is discussed.

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# Solute Movements During Volume Changes in Rat-Liver Mitochondria

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The penetration of solutes into mitochondria has two separate aspects. In the first the fraction of the mitochondrial water that is penetrated by the solute under test at equilibrium is measured. The fraction of the mitochondrial water penetrated by the solute at the same concentration as that in the medium has been termed the 'space' of that solute (Amoore, 1958) and is measured by the ratio of the concentration of the solute in the medium to that in the mitochondrial water. Measurement of these 'spaces' (see Amoore & Bartley, 1958; Amoore, 1958) gives no indication of the relative rates at which different solutes penetrate, and a direct measurement of the time course of penetration of a solute is very difficult, since the distance the solute diffuses in penetrating the mitochondrion is very small (less than 1 $\mu$) whereas the time required to isolate the mitochondria from the incubation medium is relatively long (10–15 min.). However, the composition of the fluid that enters or leaves the mitochondrion during the processes of swelling or shrinking must reflect the relative ease with which the individual solutes can pass through the membrane.

In this paper a detailed study is made of the composition of the fluid taken up by or expelled by the mitochondria when they swell or shrink. Most studies on mitochondrial swelling have been concerned only with the magnitude of the swelling and the conditions causing the swelling (e.g. the composition of the medium). Further, in most studies of swelling the increase in the volume of the mitochondria is some two or three times. With such a large increase in volume it is likely that the selectivity of the mitochondrial membrane to the passage of solutes will be greatly altered. Therefore in this work use has been made only of experiments in which the volume change of the mitochondria was relatively small.

## MATERIALS AND METHODS

The preparation of rat-liver mitochondria, the incubation techniques, separation of mitochondria from the incubation medium and determination of the dry matter of the mitochondria were according to Werkheiser & Bartley (1957).

The following methods were used for the estimation of the various solutes: sucrose (Kulka, 1956); sodium and potassium, by the lithium internal-standard flame photometer (Amoore, Parsons & Werkheiser, 1958); phosphate, Berenblum & Chain (1938) as modified by Bartley (1953); chloride, Sanderson (1952); magnesium, Mann & Yoe (1956) as modified by Amoore & Bartley (1958); manganese, Bartley, Notton & Werkheiser (1957).

In the calculation of 'spaces' of the mitochondria the water of the centrifuged mitochondrial pellet was assumed to be equal to the mitochondrial water. No allowance was made for the extraparticulate water.

*Experimental procedure.* Mitochondria, prepared according to Werkheiser & Bartley (1957), were suspended in the experimental medium and maintained at the required temperature for the time stated in the various tables. At temperatures above 0° the mitochondria were shaken in air, usually in conical flasks. At the end of the incubation time measured samples of the mitochondrial suspension were added to accurately weighed centrifuge tubes (4–5 ml. capacity). After centrifuging for 5 min. at 25 000*g* the supernatant fluid was poured as quantitatively as possible into weighed conical centrifuge tubes containing 1 ml. of 30% trichloroacetic acid. The centrifuge tubes had been weighed after the addition of the trichloroacetic acid and hence the weight and volume of this fluid were accurately known. Duplicate samples of 1 ml. of the trichloroacetic acid differed by less than 1 mg. in weight. The contents of the centrifuge tubes were mixed and the tubes again weighed. Thus the weight of solution poured off from the mitochondrial pellet was accurately known. The weight of 1 ml. of the supernatant fluid was also determined and thus the volume of supernatant fluid was also known. The inside of the centrifuge tube containing the mitochondrial pellet was carefully dried with filter paper and the weight of pellet was determined. An accurately measured quantity of 10% trichloroacetic acid (1 ml.) was added to the mitochondrial pellet and the tube and contents were again weighed. The mitochondrial pellet was broken up with a glass rod, care being taken not to remove any particles of the precipitated protein, and the tube was set aside at 0° for 12 hr. to allow thorough extraction of the mitochondrial solutes. The centrifuge tubes and contents were centrifuged at 25 000*g* for 5 min. and the supernatant fluid was decanted for analysis. The solid residue in the centrifuge tube was stirred with 5% trichloroacetic acid (about 1 ml.) and again centrifuged. The supernatant from this procedure was discarded and the washing procedure was repeated. A final wash of the mitochondrial solids was made with water and 1 ml. of water was added to the pellet. The tube containing the pellet and water was then dried at 105°; the weight of the dried pellet was determined. The water content of the original mitochondrial pellet can be determined by subtracting the weight of the dried mitochondrial

residue together with the sum of the weights of the chemically determined solutes in the wet mitochondrial pellet from the weight of the wet mitochondrial pellet. The validity of this method of determining the water content of mitochondrial pellets has been established by Werkheiser & Bartley (1957). The mitochondrial extracts and the deproteinized suspension media were analysed for their various solute contents.

The analytical data thus available for mathematical manipulation are: (1) solid, solute and water content of the mitochondrial pellet; (2) solute content, weight, density and volume of suspension medium. From (2) the concentration of solutes in which the mitochondria were suspended can be calculated. In this case the concentrations can be expressed on a molal or a molar basis. From (1) the concentrations of mitochondrial solutes can be calculated but these values can only be expressed on a molal basis since the volume of original mitochondrial fluid is not known. However, it is known that mitochondria contain about 40% of soluble protein and thus a gross error would be introduced by equating molarity with molality in this context. The deviation from ideal behaviour of the solutions used in the preparation of mitochondria may not be generally realized. Thus 0·25 M-sucrose solution is commonly used with a tacit assumption that the osmotic pressure is 0·25 osmolal. But the volume of water contained in a 0·25 M solution of sucrose is only about 91·5% of the solution volume and the osmolality is thus not 0·25 but 0·27, a difference of some 8%. In this paper all the concentrations are expressed on a molal basis. Amounts of solutes are given as quantities associated with unit dry weight of mitochondria. These are expressed as m-moles/kg. of $M$, where $M$ is the dry weight of the trichloroacetic acid-insoluble material of the mitochondria, which is within 1% of the dry weight of the mitochondria determined directly (see Werkheiser & Bartley, 1957).

*Accuracy of the chemical determinations.* Duplicate determinations agreed to better than ±2%.

*Method of calculation.* From the measured amount of each solute in the mitochondrial pellets and the measured dry weight ($M$) of the mitochondrial pellet the amounts of each individual solute associated with 1 kg. of $M$ were calculated. From the amount of water in each mitochondrial pellet the volume (l.) associated with 1 kg. of $M$ was calculated. When the amount of water/kg. of $M$ increased it was assumed that the mitochondria had swollen and when this value decreased then their volume had diminished. This assumption presumes that the partial specific volume of the dry solids is not materially altered by changes in the amount of water in the mitochondrial pellet. By comparing the amounts of solutes and water associated with unit weight of mitochondrial solids at different times an assessment can be made of changes of water and solutes occurring in the mitochondrial pellets between the two times. The changes in solute and water may be independent. For example, the solute in the mitochondrial pellet may increase without a change in the water content. With sucrose this is assumed to be due to penetration of the solute into part of the water space hitherto inaccessible. On the other hand, the water content of the mitochondria may change without a change in the total solute content of the mitochondria. Usually there is a simultaneous change in both water and solute content of mitochondria.

## RESULTS

*Solute movements during mitochondrial-volume changes in sucrose solutions.* Mitochondria do not swell appreciably in 0·25 M-sucrose at 0°, although they become increasingly permeated with sucrose (see Amoore & Bartley, 1958). However, swelling may occur in this medium when the temperature is raised to 20° and shrinking may be induced at 0° by transfer of the mitochondria from 0·25 M-sucrose to sucrose solutions of a higher osmotic pressure. In Table 1 is shown the composition of the fluid passing into the mitochondria, compared with the composition of the suspending medium, when swelling was induced by keeping at 20° for 1 hr. The amount of sucrose that penetrated the mitochondria was greater than that contained in the volume of medium that entered the mitochondria. Thus the proportion of the mitochondrial pellet permeated by sucrose increased. The data suggest that two processes occur: a penetration of sucrose alone into the mitochondrial pellet because of diffusion of this solute down the concentration gradient, and bulk movement of sucrose solution into the mitochondria. Under these conditions the water and the sucrose move in the same direction. By placing mitochondria suspended in 0·25 M-sucrose into sucrose solutions of a higher or lower osmotic pressure it is possible to induce a movement of water in the opposite direction from the net sucrose movement (Table 1, Expts. 2 and 3). Swelling or shrinking induced by transfer to solutions of different osmotic pressure increased the loss of endogenous potassium. Shrinkage by transfer to a sucrose medium of high osmotic pressure did not alter the volume of sucrose solution in the pellet that was at the same concentration as in the medium. That is, the shrinkage was extrusion of fluid from that part of the mitochondrial pellet into which the sucrose had not penetrated. It can be seen from Table 1 that all the potassium content of the pellet and an equivalent amount of anion must be allotted to the sucrose-free water of the pellet to obtain approximately iso-osmolality with the medium. On resuspending the 'hyperosmotic' mitochondria in 0·25 M-sucrose the uptake of water was greater than that lost by the previous shrinkage, and the volume occupied by iso-osmolal sucrose increased by 45% whereas the sucrose-free water increased by about 15%. Again, all the potassium must be present in the sucrose-free water of the mitochondria for this to have the same osmotic pressure as the medium. The loss in $K^+$ ions that occurred on transfer from solutions of higher osmolality to 0·25 M-sucrose is as would be predicted if all the potassium was contained in the sucrose-free water. The reciprocal relationship

Table 1. *Movement of water and solutes with volume changes of mitochondria suspended in sucrose solutions*

The total quantity of each solute, the water content and the dry matter of the mitochondrial pellets were determined as described in the text. Duplicates of chemical determinations agreed better than ±2%. Wet mitochondrial pellets weighed 100–150 mg. and the dry matter was 30–50 mg. Duplicate weighings agreed within 0·2 mg. Concentrations are calculated from the measured quantities of solutes and water by determining how much of each solute was contained in 1 kg. of water. The amount of solute taken up or extruded is calculated from the chemically determined amounts in the pellets. The composition of the solution taken up or extruded is calculated from the differences in the determined solute and water contents of the two mitochondrial pellets. The sucrose-containing water is calculated from the sucrose content of the mitochondrial pellet as that fraction of the pellet water necessary to contain the pellet sucrose at the same concentration as in the medium.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Kept at 20° for 60 min. | 1·75 | 0·46 | 2·2 | 0·39 | $K^+$ | −3·1 | 35·2 | — | 2·6 |
| | | | | | | $Cl^-$ | 7·7 | 1·76 | 20 | 1·6 |
| | | | | | | Sucrose | 108 | 191 | 285 | 241 |
| | | | | | | $Mg^{2+}$ | −1·7 | 18 | — | — |
| 2 | Suspension prepared in 0·25 M-sucrose then sufficient 2·5 M-sucrose was added to make the final concn. of sucrose about 0·5 M. Kept at 0° for 15 min. | 1·4 | 0·64 | 1·35 | 0·27 | $K^+$ | −13 | 41·4 | 31 | 0 |
| | | | | | | $Cl^-$ | −1·29 | 0·63 | 3 | 0 |
| | | | | | | Sucrose | 336 | 179 | — | 541 |
| 3 | Mitochondrial pellet in approx. 0·5 M-sucrose resuspended in approx. 0·25 M-sucrose; kept for 15 min. at 0° | 1·35 | 0·27 | 1·96 | 0·31 | $K^+$ | −29·2 | 44·1 | — | 0 |
| | | | | | | $Cl^-$ | 0 | 0 | — | 0 |
| | | | | | | Sucrose | −200 | 433 | — | 281 |

Table 2. *Movement of water and solutes with volume changes of mitochondria suspended in sucrose solution containing small amounts of potassium chloride*

For method of calculation see Table 1 and text.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Kept in 0·25 M-sucrose + 0·01 M-KCl for 15 min. at 0° | 2·18 | 0·65 | 2·6 | 0·43 | $K^+$ | 24·8 | 26·5 | 124 | 12·5 |
| | | | | | | $Cl^-$ | 23·9 | 4·7 | 119 | 10·3 |
| | | | | | | Sucrose | 64 | 197 | 320 | 240 |
| | | | | | | $Mg^{2+}$ | −2·4 | 11·4 | — | — |

Table 3. *Movement of water and solutes with volume changes of mitochondria suspended in sucrose solutions containing small amounts of magnesium chloride*

For method of calculation see Table 1 and text.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Kept in 0·25 M-sucrose + 2 mM-$MgCl_2$ for 15 min. at 0° | 2·18 | 0·65 | 2·16 | 0·55 | $K^+$ | −19 | 26·5 | 146 | 2·6 |
| | | | | | | $Cl^-$ | 9·4 | 4·7 | — | 5·2 |
| | | | | | | Sucrose | 2·0 | 197 | — | 260 |
| | | | | | | $Mg^{2+}$ | 12·8 | 11·4 | — | 1·9 |
| 2 | Kept in 0·25 M-sucrose + 5 mM-$MgCl_2$ for 15 min. at 0° | 2·18 | 0·65 | 2·02 | 0·51 | $K^+$ | −18·9 | 26·5 | 63 | 2·5 |
| | | | | | | $Cl^-$ | 27·9 | 4·7 | — | 13·8 |
| | | | | | | Sucrose | −66 | 197 | 186 | 248 |
| | | | | | | $Mg^{2+}$ | 27·6 | 11·4 | — | 6·4 |

between sucrose and potassium content of mitochondrial preparations isolated in sucrose solutions has already been pointed out by Amoore & Bartley (1958).

Swelling or shrinking was also induced in mitochondria suspended in 0·25 M-sucrose solution at 0° by the addition of comparatively small amounts of salts (Tables 2 and 3).

It is not clear why swelling should occur when small amounts of KCl are added to the medium. Amoore & Bartley (1958) have shown that KCl, added to a mitochondrial suspension in sucrose solution, rapidly equilibrates with the sucrose-containing fluid of the mitochondrial pellet. This is demonstrated in this experiment by the large increase in the potassium content of the mitochondrial pellet. The sucrose-containing water of the pellet increased from 2·18 to 2·6 l./kg. of *M* whereas the sucrose-free water diminished from 0·65 to 0·43 l./kg. of *M*. This type of swelling should be contrasted with the mitochondrial shrinkage that occurred in sucrose solution of high osmolality, where all the loss in water was contributed by the sucrose-free fluid.

*Shrinkage in the presence of magnesium chloride.* Mitochondria shrank slightly when approx. 2 mM-$MgCl_2$ was added to a suspension in 0·25 M-sucrose solution. The diminution in volume was almost all due to a loss in the sucrose-free water. However, when the $MgCl_2$ added was 5 mM there was a loss of both the sucrose-containing and the sucrose-free water. With both magnesium concentrations the loss of potassium was approximately equal to the potassium content of the sucrose-free water lost (Table 3). It is assumed here that all the endogenous potassium is contained in the sucrose-free water of the pellet. In contrast, the potassium lost by the mitochondria, when shrinkage due to exposure to hyperosmotic sucrose solutions occurred, was only about 30% of that expected by the loss of the sucrose-free water.

*Shrinkage in the presence of manganese chloride.* Manganese ions also produced a shrinkage of mitochondria but the effect was much larger than with magnesium (Table 4). Thus 1 mM-$Mn^{2+}$ ions produced about the same shrinkage as 2 mM-$Mg^{2+}$ ions. With this low concentration of $Mn^{2+}$ ions the shrinkage was largely in the sucrose-free water, but with higher concentrations both sucrose-containing and sucrose-free water decreased. The potassium loss was constant and independent of the manganese concentration added. It was about twice that expected by the shrinkage of the sucrose-free water.

*Shrinkage in the presence of hydrochloric acid.* As with $MnCl_2$ and $MgCl_2$ the addition of HCl (0·5 mM) caused a diminution in the sucrose-free water of the pellet, and as with these two salts the

Table 4. *Movement of water and solutes with volume changes of mitochondria suspended in sucrose solutions containing small amounts of manganous chloride*

For method of calculation see Table 1 and text.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Kept in 0·25 M-sucrose + 1 mM-$MnCl_2$ for 15 min. at 0° | 2·18 | 0·65 | 2·14 | 0·52 | $K^+$ | −32·3 | 26·5 | 190 | 2·9 |
| | | | | | | $Cl^-$ | −0·3 | 4·7 | 2 | 2·0 |
| | | | | | | Sucrose | −10·0 | 197 | 59 | 257 |
| | | | | | | $Mg^{2+}$ | −6·3 | 11·4 | 37 | — |
| | | | | | | $Mn^{2+}$ | 25·6 | — | — | 0·1 |
| 2 | Kept in 0·25 M-sucrose + 2 mM-$MnCl_2$ for 15 min. at 0° | 2·18 | 0·65 | 1·82 | 0·49 | $K^+$ | −31·7 | 26·5 | 61 | 2·8 |
| | | | | | | $Cl^-$ | 4·2 | 4·7 | — | 4·1 |
| | | | | | | Sucrose | −92 | 197 | 176 | 256 |
| | | | | | | $Mg^{2+}$ | −4 | 11·4 | 18 | — |
| | | | | | | $Mn^{2+}$ | 43 | — | — | 0·55 |
| 3 | Kept in 0·25 M-sucrose + 5 mM-$MnCl_2$ for 15 min. at 0° | 2·18 | 0·65 | 1·73 | 0·47 | $K^+$ | −32·8 | 26·5 | 52 | 2·8 |
| | | | | | | $Cl^-$ | 20·6 | 4·7 | — | 10·4 |
| | | | | | | Sucrose | −128 | 197 | 203 | 250 |
| | | | | | | $Mg^{2+}$ | −11·3 | 11·4 | 18 | — |
| | | | | | | $Mn^{2+}$ | 60 | — | — | 2·3 |

Table 5. *Movement of water and solutes with volume changes of mitochondria suspended in sucrose solutions containing small amounts of hydrochloric acid*

For method of calculation see Table 1 and text.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Kept in 0·25 M-sucrose + 0·5 mM-HCl for 15 min. at 0° | 2·18 | 0·65 | 2·28 | 0·4 | $K^+$ | −36·6 | 26·5 | 244 | 2·4 |
| | | | | | | $Cl^-$ | 3·8 | 4·74 | 25 | 0·4 |
| | | | | | | Sucrose | 17 | 197 | — | 253 |
| | | | | | | $Mg^{2+}$ | −3·6 | 11·4 | 24 | — |
| 2 | Kept in 0·25 M-sucrose + 1 mM-HCl for 15 min. at 0° | 2·18 | 0·65 | 2·31 | 0·39 | $K^+$ | −42 | 26·5 | 323 | 2·7 |
| | | | | | | $Cl^-$ | −4·3 | 4·7 | 33·8 | 0·7 |
| | | | | | | Sucrose | 37 | 197 | — | 258 |
| | | | | | | $Mg^{2+}$ | −3·3 | 11·4 | 25·4 | — |
| 3 | Kept in 0·25 M-sucrose + 2 mM-HCl for 15 min. at 0° | 2·18 | 0·65 | 2·04 | 0·41 | $K^+$ | −46 | 26·5 | 121 | 2·7 |
| | | | | | | $Cl^-$ | 0·7 | 4·7 | — | 1·2 |
| | | | | | | Sucrose | −35 | 197 | 92 | 258 |
| | | | | | | $Mg^{2+}$ | −6 | 11·4 | 15·9 | — |

Table 6. *Movement of water and solute with volume changes of mitochondria suspended in a mixture of sucrose and potassium chloride solution*

'Treatment of mitochondria' describes the history of the mitochondrial preparation up to the 'initial time' (at this time a sample of the mitochondrial suspension was removed and the mitochondria were sedimented for analysis: the analyses are given under 'Initial water content of mitochondrial pellet' and under 'Concn. of solute', 'Initial water of pellet') and its subsequent treatment during the experimental period. For method of calculation see Table 1 and text.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Mitochondria prepared in 0·25 M-sucrose were added to KCl soln. to give suspension medium containing 0·1 M-KCl and 0·05 M-sucrose (initial time) then kept at 0° for 60 min. | 1·74 | 0·65 | 1·81 | 0·69 | $K^+$ | 23 | 106 | 209 | 108 |
| | | | | | | Sucrose | 3·6 | 36·3 | 32·7 | 49·9 |
| 2 | As above but warmed to 21° for 5 min. | 1·74 | 0·65 | 1·80 | 0·75 | $K^+$ | 17·2 | 106 | 169 | 108 |
| | | | | | | Sucrose | 8·0 | 36·3 | 19·4 | 49·9 |
| 3 | As above but warmed to 21° for 25 min. | 1·74 | 0·65 | 2·41 | 0·74 | $K^+$ | 133 | 106 | 175 | 108 |
| | | | | | | Sucrose | 33·1 | 36·3 | 43·5 | 49·9 |
| 4 | Mitochondria prepared in sucrose solution were added to KCl soln. to give approx. 0·1 M-KCl and 0·05 M-sucrose in suspending medium. Kept for 5 min. at 0° (initial time) then maintained for further 55 min. at 0° | 3·89 | 0 | 4·36 | 0 | $K^+$ | 28 | 124 | 59·5 | 115 |
| | | | | | | $Cl^-$ | 136 | 109 | 290 | 113 |
| | | | | | | Sucrose | 23 | 50 | 49 | 49·7 |
| | | | | | | $Mg^{2+}$ | −5·5 | 3·7 | — | 0·25 |
| | | | | | | $PO_4^{3-}$ | −24·1 | 19·3 | — | 0·6 |
| 5 | Mitochondria prepared in sucrose soln. as above but maintained for 35 min. at 0° (initial time) then kept at 23·8° for 22·5 min. | 4·20 | 0 | 6·11 | 0·91 | $K^+$ | 250 | 119 | 119 | 110 |
| | | | | | | $Cl^-$ | 272 | 115 | 130 | 108 |
| | | | | | | Sucrose | 96 | 50 | 45·5 | 50 |
| | | | | | | $Mg^{2+}$ | 1·2 | 1·9 | 0·57 | 0·26 |
| | | | | | | $PO_4^{3-}$ | −8·1 | 12·5 | — | 0·71 |
| 6 | Mitochondria prepared in 0·25 M-sucrose with added KCl to give final concn. 0·04 M (initial time) were then incubated for 60 min. at 20° | 2·11 | 0·54 | 3·87 | 0 | $K^+$ | 1 | 80·5 | 0·79 | 38 |
| | | | | | | $Cl^-$ | 78·9 | 33·1 | 62 | 42 |
| | | | | | | Sucrose | 439 | 179 | 345 | 232 |

Table 7. *Movement of water and solutes with volume changes of mitochondria suspended in potassium chloride solutions*

For method of calculation see Table 1 and text. For other details see Table 6.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Prepared in 0·25 M-sucrose, suspended in 0·125 M-KCl at 0° (initial time) and then kept at 0° for 15 min. | 1·37 | 0·67 | 2·61 | 0 | $K^+$ | 267·4 | 41·4 | 46·9 | 127 |
| | | | | | | $Cl^-$ | 267·4 | 0·63 | 461 | 125 |
| | | | | | | Sucrose | −326·9 | 179 | — | 12·4 |
| 2 | Prepared in 0·25 M-sucrose, suspended in 0·125 M-KCl at 0° (initial time) and then kept at 0° for 8 hr. | 2·36 | 0·18 | 2·61 | 0·44 | $K^+$ | 93 | 122 | 182 | 122 |
| | | | | | | $Cl^-$ | 106 | 68·5 | 207 | 119 |
| | | | | | | Sucrose | 3·1 | 8·2 | 6·1 | 9·0 |
| | | | | | | $PO_4^{3-}$ | −20·6 | 37·1 | — | — |
| | | | | | | $Mg^{2+}$ | −4·0 | 9·2 | — | — |
| 3 | Prepared in 0·25 M-sucrose, sucrose soln. (2·5 M) added to suspension to make sucrose concn. 0·5 M; centrifuged mitochondria (initial time) and resuspended pellet in 0·125 M-KCl was kept at 0° for 15 min. | 1·29 | 0·33 | 3·02 | 0·25 | $K^+$ | 358 | 44·1 | 217 | 127 |
| | | | | | | $Cl^-$ | 325 | 0 | 213 | 125 |
| | | | | | | Sucrose | −646 | 433 | — | 18·6 |
| 4 | Prepared in 0·25 M-sucrose, KCl soln. (1·25 M) added to suspension to make KCl concn. 0·125 M (initial time) and resuspended pellet in 0·125 M-KCl was kept at 0° for 15 min. | 1·32 | 0·45 | 2·69 | 0 | $K^+$ | 53 | 174 | 59 | 127 |
| | | | | | | $Cl^-$ | 51 | 120 | 57 | 131 |
| | | | | | | Sucrose | −290 | 179 | — | 6·8 |
| 5 | Prepared in 0·25 M-sucrose, sucrose soln. (2·5 M) added to made concn. 0·5 M, centrifuged and resuspended in 0·125 M-KCl (initial time) and then kept at 0° for 15 min. | 1·78 | 0·49 | 7·0 | 0 | $K^+$ | 858 | 18·7 | 181 | 129 |
| | | | | | | $Cl^-$ | 885 | 0 | 187 | 128 |
| | | | | | | Sucrose | −435 | 221 | — | 8·9 |
| 6 | Prepared in 0·25 M-sucrose, KCl soln. (1·25 M) added to make 0·125 M-KCl, centrifuged (initial time), suspended in 0·125 M-KCl and then at 0° for 15 min. | 1·49 | 0·53 | 6·9 | 0·42 | $K^+$ | 840 | 44·7 | 159 | 129 |
| | | | | | | $Cl^-$ | 909 | 0 | 171 | 124 |
| | | | | | | Sucrose | −350 | 198 | — | 7·7 |

'sucrose space' was little affected by further increase in the HCl content of the medium (Table 5). The sucrose-containing water was not diminished by the lower concentration of HCl (cf. Tables 3 and 4 with $Mg^{2+}$ and $Mn^{2+}$ ions); instead some slight increase occurred. The higher concentrations produced a contraction of the sucrose-containing water similar to that which occurred with $MnCl_2$ and $MgCl_2$.

*Volume changes of mitochondria in sucrose–potassium chloride mixtures.* Usually storage at 0° or at room temperature in a solution containing sucrose and KCl of 0·25 osmolal caused a swelling. The magnitude of this varied (compare Expts. 1–3 with Expts. 4–6, Table 6). When the water content of the mitochondria reached about double that of normal mitochondria isolated in 0·25 M-sucrose (about 2·0 l. of water/kg. of *M*), they became totally permeable to sucrose. It was only in exceptional circumstances that the fluid taken up by the mitochondria corresponded in composition with the suspending medium (e.g. Expt. 5, Table 6). This swelling, which corresponds with that described by Leaf (1956), occurred when the water content of the mitochondria exceeded 4·0 l./kg. of *M* and when the sucrose-containing water occupied the whole of the mitochondrial water. Below this degree of swelling the mitochondria retained some degree of selectivity towards the composition of the fluid passing into or out of them. For example, in Expt. 4, Table 6, there is a marked discrepancy in the amount of anion and cation taken up by the mitochondria. It is not at all clear, for example, what is the compensating ion making for electrical neutrality in this experiment. The pH of the internal fluid of the mitochondria is not known but it seems unlikely that it would differ greatly from neutrality. There must therefore be a lowering of the pH of the internal fluid of the mitochondria, the magnitude of which will depend on the buffering capacity of the internal proteins. The uptake of chloride was partly compensated for by loss of phosphate, but there remained the equivalent of some 70 m-moles of chloride to be compensated for. This in unbuffered solution would lower the pH to between 1 and 2. Experiments in which the mitochondria were directly titrated with HCl (Amoore & Bartley, 1958; Bartley & Amoore, 1958) showed that the mitochondria will buffer so that the addition of 1 $\mu$mole of HCl to 27 mg. of mitochondrial dry matter in 1 ml. produced a pH drop of only 0·3 unit. This absorption of $H^+$ ions may be a reversal of the acid secretion which occurred when $MnCl_2$ was added to the mitochondrial suspensions.

*Volume and solute changes of mitochondria suspended in potassium chloride solutions.* In these experiments the mitochondria were prepared in

Table 8. *Movement of water and solutes with volume changes of mitochondria suspended in various saline media*

For method of calculation see Table 1 and text.

| Expt. no. | Treatment of mitochondria | Initial water content of mitochondrial pellet (l./kg. of *M*) | | Final water content of mitochondrial pellet (l./kg. of *M*) | | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | Prepared in 0·25 M-sucrose and suspended in a medium containing 24·2 mM-$K^+$, 6·5 mM-$Na^+$, 22 mM-$Cl^-$, 4 mM-$PO_4^{3-}$, 5 mM-α-oxoglutarate, 5 mM-AMP; incubated for 10 min. at 25° | 2·93 | | 3·63 | | $Na^+$ 4·1<br>$K^+$ −3·0 | 14·6<br>61·5 | 5·9<br>— | 8·8<br>29·6 |
| 2 | Prepared in 0·25 M-sucrose and then suspended in medium at 0° for 17 min. (concentrations of solutes in medium are given under 'Supernatant water') | 2·68 | | 3·29 | | $K^+$ 30<br>$Na^+$ −4·3<br>$Cl^-$ 21<br>$PO_4^{3-}$ 12·5<br>Sucrose 20<br>$Mg^{2+}$ 1 | 99<br>30·4<br>63·1<br>39·4<br>43·7<br>7·2 | 49·2<br>—<br>34·5<br>20·5<br>32·8<br>1·6 | 88<br>27·5<br>67·0<br>18·5<br>46·4<br>5·0 |
| | | Sucrose-containing water | Sucrose-free water | Sucrose-containing water | Sucrose-free water | | | | |
| | | 2·52 | 0·16 | 2·96 | 0·33 | | | | |

0·25 M-sucrose solution and after various treatments were suspended in 0·125 M-KCl. Since the particles were not washed with the KCl solution a small amount of sucrose (6–12 m-molal) remained in the suspending fluid. This enabled an estimate to be made of the sucrose-containing and sucrose-free water. Resuspension of the mitochondria in 0·125 M-KCl at 0° (Expt. 1, Table 7) caused a 28 % increase in the water content and rendered the pellet completely permeable to sucrose. The amounts of $K^+$ and $Cl^-$ ions passing into the pellet showed that these could freely diffuse throughout the water of the pellet and their uptake was limited to the amount contained in the volume of solution that entered the mitochondria. In some experiments (Table 7) the impermeability to sucrose was not completely abolished by transfer to 0·125 M-KCl solution. As the preparation swelled when stored at 0° there was an increase in volume of both the sucrose and sucrose-free water. The chloride, which was taken up in greater amount than $K^+$ ions, was now evenly distributed throughout the pellet water, but there was a net overall excess of $K^+$ ions, resulting in the water of the pellet having a $K^+$ ion concentration of 142 m-molal. It seems unlikely that all the $K^+$ ions can be in free solution in the pellet water. It is possible that the swelling of the mitochondria resulted in an unfolding of protein structures with the formation of new cation-binding sites on the protein. It appears from Expts. 3 and 4 (Table 7) that the rate of movement of water must be faster than that of sucrose and the mitochondrial membrane must retain some semipermeable properties, since swelling of the sucrose space occurred in both these experiments. Apparently the treatment given to the mitochondria described in Expts. 5 and 7 (Table 7) greatly increased the permeability of the mitochondria to KCl and water without greatly altering that to sucrose. This resulted in the swelling shown in these two experiments.

*Movement of water and solutes with volume changes of mitochondria in saline media.* Mitochondria prepared in 0·25 M-sucrose solution and suspended in the medium shown in Table 8 (Expt. no. 2) swelled at 0°. Both the sucrose and the sucrose-free water increased but the percentage increase was greater in the sucrose-free water. It can be seen that the composition of the fluid taken up by the mitochondria is very different from that of the incubation medium, showing that the mitochondrial membrane still retains its differential permeability.

*Effect of calcium and magnesium on sodium, potassium and water movement in mitochondria.* In these experiments the mitochondria were suspended in the type of medium in which maximum rates of uptake of $O_2$ are expected to occur. The mitochondria were added to the medium at 0° and subsequently warmed to 25°. As shown in Table 9, calcium and magnesium had opposite effects on the initial potassium content of the pellet. Calcium caused a loss of potassium from the mitochondria, whereas magnesium caused the endogenous potassium to be retained better than in its absence. Both calcium and magnesium caused a slight swelling of the mitochondrial pellet during the initial period at 0° but on subsequent incubation at 25° magnesium prevented the swelling that occurred without magnesium or in the presence of calcium. Magnesium also lessened the uptake of sodium from the medium at 0°, whereas calcium had little effect. When incubated at 25° the opposite roles of calcium and magnesium again became apparent since the presence of calcium increased the uptake of sodium, whereas the presence of magnesium decreased it. The uptake of sodium in all three cases was against a concentration gradient but the nominal concentration of sodium in the solution that was taken up in the three cases was highest with magnesium and lowest in the absence of both calcium and magnesium. Similar observations

Table 9. *Effect of calcium and magnesium on solute and water movement in mitochondria*

For method of calculation see Table 1 and text. The mitochondria were prepared in sucrose and suspended in a medium containing 24·2 mM-$K^+$ ions, 22 mM-$Cl^-$ ions, 3 mM-$PO_4^{3-}$ ions, 5 mM-α-oxoglutarate and 5 mM-AMP; in Expt. 2 a further addition of 2·5 mM-$Ca^{2+}$ ions was made and in Expt. 3 2·5 mM-$Mg^{2+}$ ions. Incubation was for 10 min. at 25°.

| Expt. no. | Initial water content of mitochondrial pellet (l./kg. of *M*) | Final water content of mitochondrial pellet (l./kg. of *M*) | Solute | Solute taken up or extruded (m-moles/kg. of *M*) | Concn. of solute (m-molal) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Initial water of pellet | Soln. extruded or taken up | Supernatant water |
| 1 | 2·93 | 3·63 | $Na^+$ | 4·1 | 14·6 | 5·9 | 8·8 |
| | | | $K^+$ | −30 | 61·5 | — | 29·6 |
| 2 | 3·04 | 3·60 | $Na^+$ | 7·3 | 12·9 | 13 | 8·8 |
| | | | $K^+$ | 0 | 46·4 | 0 | 29·6 |
| 3 | 3·03 | 3·17 | $Na^+$ | 2·5 | 9·7 | 17·8 | 8·8 |
| | | | $K^+$ | −7 | 65·7 | — | 29·6 |

have been made on the permeability of red-cell 'ghosts' by J. F. Hoffman, D. C. Tosterson & R. Whittam (unpublished work).

## DISCUSSION

Leaf (1956) has shown that under certain conditions the fluid entering the tissues in the process of swelling was a solution approximately iso-osmotic with the medium. The distribution of the imbibed fluid within the cell is not known, but it is reasonable to suppose that it will not be confined to the extramitochondrial fraction of the cell. If the fluid taken up is evenly distributed throughout the cell volume, then the intracellular mitochondria cannot be exhibiting the same permeability characteristics as the isolated mitochondria since these swelled only with uptake of unaltered medium, when they had been subjected to gross osmotic damage. Yet it seems unlikely that the permeability of the isolated mitochondria would be less than when they were in the cell. On the other hand, if the swelling described by Leaf (1956) does not involve the mitochondrial compartment of the cell then no deductions can be made about the state of the mitochondrial membrane within the cell.

The passive passage of a solute through a membrane is dependent on the driving force supplied by the differences in the external and internal concentrations. The ease with which this transfer of solute takes place is dependent on the permeability of the membrane to the particular solute. Thus if the volume of the mitochondria is increased we should expect solutes to pass into the mitochondria in the same proportion as in the medium if the mitochondrial membrane is freely permeable to all these solutes. As seen by the data presented, this is seldom realized. The ratio of concentration of solute in water taken up/concentration of solute in the medium is a measure of the permeability of the mitochondrial membrane to the solute. This value will be 1 if the membrane is freely permeable to the solute and 0 if the membrane is completely impermeable to the solute. It is important to realize that this ratio is independent of the absolute concentration of solute in the medium. The value can be expressed as a percentage of the water permeability by multiplying the values by 100. A similar argument can be applied to the shrinkage of mitochondria where concentration of solute in water extruded/concentration of water in mitochondrial medium measures the permeability of the membrane to the internal solutes. If the value for the permeability is above 100 this suggests that either some form of absorption is occurring, and all the solute passing into the mitochondria is not in solution, or the passage of the solute has been facilitated by some metabolic process. It is clear that the estimate of the permeability is liable to be too low (*a*) when there is an active extrusion process for any solute working in the opposite direction to the bulk solute flow, and (*b*) when there is a concentration gradient of the solute in the opposite direction to the bulk solute flow. When for any reason the net solute movement is in the opposite direction to the bulk solution flow the solute movement is given a negative sign. Any results where (*a*) or (*b*) can be shown to occur or where the sign of the solute movement is negative have not been considered in the assessment of the permeability of the mitochondrial membrane. From Table 10 it can be seen that the permeability of the mitochondria to sucrose is variable. Usually sucrose does not penetrate the mitochondrial membrane as readily as does water, the mean percentage permeability being 78 %. The passage of sucrose through the mitochondrial membrane does not render the membrane freely permeable to sucrose, as shown by the comparatively small amount of this solute extruded on shrinkage. The

Table 10. *Comparison of sucrose and water passage through the mitochondrial membrane*

The passage of water is taken as 100 %; the values of the sucrose movement are considered to be the same as the water movement if the concentration in the solution taken up or extruded is the same as that in extramitochondrial fluid or intramitochondrial fluid respectively.

| Expt. no. | Experimental conditions | Permeability (%) compared with water | Volume change of mitochondria (%) |
|---|---|---|---|
| 1 | 20°; 60 min. in 0·25 M-sucrose | 118 | +17 |
| 2 | 0°; 15 min. in 0·25 M-sucrose + 0·01 M-KCl | 133 | +7 |
| 3 | 0°; 15 min. in 0·25 M-sucrose + 5 mM-$MgCl_2$ | 95 | −10·5 |
| 4 | 0°; 15 min. in 0·25 M-sucrose + 1 mM-$MnCl_2$ | 30 | −6 |
| 5 | 0°; 15 min. in 0·25 M-sucrose + 2 mM-$MnCl_2$ | 90 | −18 |
| 6 | 0°; 15 min. in 0·25 M-sucrose + 5 mM-$MnCl_2$ | 103 | −22 |
| 7 | 0°; 15 min. in 0·25 M-sucrose + 2 mM-HCl | 46·5 | −14 |
| 8 | 0°; 60 min. in 0·05 M-sucrose + 0·1 M-KCl | 65 | +5 |
| 9 | 21°; 5 min. in 0·05 M-sucrose + 0·1 M-KCl | 100 | +7 |
| 10 | 21°; 25 min. in 0·05 M-sucrose + 0·1 M-KCl | 87 | +31·5 |
| 11 | 0°; 17 min. in medium described in Table 8 (Expt. no. 2) | 71 | +23 |

results of Expts. 8 and 9 (Table 10) suggest that the permeability of the mitochondrial membrane to sucrose is diminished by increase in temperature. The effect of increasing manganese concentration increases the shrinkage of the mitochondria and increases the permeability of the mitochondria to sucrose. In most of the experiments given in Table 10 the movement of potassium was also measured. These results (Table 11) show that potassium permeability was uniformly larger than 100%. Since most of these experiments were carried out at 0° it seems that these high values are the results of the participation of adsorption processes during the exchange of potassium between mitochondria and medium. Adsorption of potassium has already been demonstrated by Amoore & Bartley (1958). In the one experiment (Expt. 10, Table 11) where relative impermeability to potassium could be demonstrated, the percentage permeability was less than that of sucrose and very similar to that of chloride (see also Amoore, 1958).

The percentage permeability of sodium is generally similar to that of sucrose and chloride (Amoore & Bartley, 1958; Bartley & Amoore, 1958), but this can vary with the presence of bivalent cations in the medium. In a medium suitable for oxidative phosphorylation (Table 9) the percentage permeability of sodium was 67, but on the addition of 2·5 mM-calcium it rose to 148 and with 2·5 mM-magnesium it became 202. It seems probable that in this case the effect of the bivalent cations is to liberate binding sites suitable for the attachment of sodium.

All these experiments illustrate the variation in the properties of adsorption and permeability of the mitochondrial membrane that can result from changes in the composition of the suspending medium. It is to be supposed that changes in the metabolic properties of the mitochondria will follow from these variations. It would seem particularly important when studies are being made on mitochondrial swelling to give a full description of the water and solute content of the starting material, since the degree of swelling or shrinking will largely depend on this. This information is very seldom given and the situation is further complicated by the frequent use of optical methods to measure swelling, which are usually not calibrated against the changes in the water content of the mitochondria and which ignore the possible contributions to the changes in light-scattering of the changes in refractive index of the intramitochondrial solution. Tedeschi & Harris (1955), from light-scattering studies, had concluded that mitochondria were impermeable to sucrose and behaved as osmometers in solutions of this solute. The work of Werkheiser & Bartley (1957) and Amoore & Bartley (1958) showed by direct measurement that sucrose did in fact penetrate liver mitochondria to a variable extent, usually some 50–60% of their volume. Recknagel & Malamed (1958), who first doubted the validity of the observations of Werkheiser & Bartley (1957), have since confirmed them (Malamed & Recknagel, 1959). They conclude that the fraction of the mitochondrial volume into which sucrose does not penetrate responds as expected by volume changes when the concentration of sucrose in the suspending medium is varied. This fact was already apparent in the results of Werkheiser & Bartley (1957) and is demonstrated in this paper. However, it is not only the sucrose-free space of the mitochondria that changes in volume in response to changes in the external medium. Bivalent cations may cause shrinkage of the sucrose-containing water as well as the sucrose-free space and, in mitochondria which have been penetrated completely by sucrose, volume changes occur in response to changes in the medium. It is still not clear what significance the changes in mitochondrial permeability *in vitro* have for the understanding of the behaviour of mitochondria *in vivo*. It is an attractive theory that permeability changes may be linked with the control of the rate and direction of cellular metabolism. There is as yet no evidence contradicting this supposition but the possibility must also be

Table 11. *Comparison of passage of potassium chloride and water through the mitochondrial membrane*

| Expt. no. | Experimental conditions | K permeability (%) compared with water | Cl permeability (%) compared with water | Volume change of mitochondria (%) |
|---|---|---|---|---|
| 1 | 0°; 15 min. in 0·25 M-sucrose + 0·01 M-KCl | 1000 | 1000 | +7 |
| 2 | 0°; 15 min. in 0·25 M-sucrose + 5 mM-$MgCl_2$ | 237 | — | −9·5 |
| 3 | 0°; 15 min. in 0·25 M-sucrose + 1 mM-$MnCl_2$ | 720 | — | −6 |
| 4 | 0°; 15 min. in 0·25 M-sucrose + 2 mM-$MnCl_2$ | 230 | — | −18 |
| 5 | 0°; 15 min. in 0·25 M-sucrose + 5 mM-$MnCl_2$ | 196 | — | −22 |
| 6 | 0°; 15 min. in 0·25 M-sucrose + 2 mM-HCl | 456 | — | −14 |
| 7 | 0°; 60 min. in 0·05 M-sucrose + 0·1 M-KCl | 193 | — | +5 |
| 8 | 21°; 5 min. in 0·05 M-sucrose + 0·1 M-KCl | 156 | — | +7 |
| 9 | 25°; 5 min. in 0·05 M-sucrose + 0·1 M-KCl | 162 | — | +31·5 |
| 10 | 0°; 15 min. in medium described in Table 8 (Expt. no. 2) | 55·9 | 51·4 | +27 |

considered that the permeability characteristics merely reflect the chemical structure of the membrane and are not in themselves of importance as mechanisms regulating metabolism.

## SUMMARY

1. The water and solute content of rat-liver mitochondria have been measured during swelling or shrinking of the particles.

2. When mitochondria swell in 0·25 M-sucrose solution there is an increase in the fraction of mitochondrial water penetrated by sucrose. The shrinkage due to transfer of mitochondria from 0·25 M-sucrose to solutions of a higher osmoticity is mainly due to the loss of water from the sucrose-free space.

3. In mitochondria partially permeated by sucrose it is necessary to assume for osmotic equilibrium that the endogenous potassium is solely within a compartment separate from the sucrose.

4. When magnesium or manganese cause a shrinkage of mitochondria there is a loss of potassium that is never greater than that expected from the volume change of the sucrose-free water.

5. When mitochondria have swollen to a state when they contain more than 4 l. of water/kg. of solids they become completely permeable to sucrose and in further swelling the volume changes are due to an uptake of the suspending medium. Below 4 l. of water/kg. of solids the mitochondria are able partially to exclude some components of the medium.

6. Calcium and magnesium have antagonistic effects on mitochondria. Calcium increases the uptake of sodium and increases the loss of potassium, whereas magnesium decreases both these processes.

7. The problems of mitochondrial permeability are discussed.

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# Selective Determination of Sugars Manifesting Enediol Isomerism by means of Reaction with Tetrazolium

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2:3:5-Triphenyltetrazolium chloride in aqueous alkaline solution (Wallenfels, 1950) has been used as a selective spray reagent on paper for the detection of sugars manifesting 1:2-enediol isomerism (Bell & Dedonder, 1954; Feingold, Avigad & Hestrin, 1956; cf. also Barker, Bourne, Grant & Stacey, 1956; Haq & Whelan, 1956; Côté, 1959; O'Donnell & Percival, 1959). It has been noted by Bacon (1959) that the reaction of 2:5-diphenyl-3-(4-styrylphenyl)tetrazolium chloride yields a deeper colour than does the reaction with triphenyltetrazolium chloride. In the present paper a selective quantitative assay of sugars, manifesting enediol isomerism, is based on reaction with these tetrazolium reagents and is used in particular for the selective determination of oligofructosides bearing a substituent on C-6 (compounds of the type of 6-R-fructose) in the presence of those bearing a substituent on C-1 (compounds of the type of 1-R-fructose).

## METHOD

*Reagents.* 2:3:5-Triphenyltetrazolium chloride (TTC) was an aqueous 1% (w/v) solution. This keeps for about one week at room temperature in the dark. TTC (Schwarz Inc., New York) gave a deeper colour on interaction with sugars than a preparation supplied by National Biochemicals Co., Cleveland, Ohio, U.S.A., and was used throughout the present investigation. 2:5-Diphenyl-3-(4-styrylphenyl)tetrazolium chloride (DSTC) (May and Baker, Ltd., Dagenham) was an aqueous 0·2% solution. This keeps at room temperature in the dark for at least one month.

Other reagents were: sodium hydroxide (0·5N); borate (1 vol. of 0·2N-NaOH was mixed with 1 vol. of 0·2N-sodium tetraborate); acidic methanol [10% (w/v) acetic acid in methanol]; acidic propanol [10% (w/v) acetic acid in propan-2-ol].

*Procedure.* Aqueous sugar solution is deproteinized if necessary by addition of 4% (w/v) trichloroacetic acid. The cleared solution is adjusted with N-alkali to pH 7·0. If a zinc–barium mixture is used for this purpose, excess of barium must be avoided.

To a test tube containing 0·7 ml. of neutralized sugar solution in water are added, with mixing, 0·1 ml. of tetrazolium reagent (TTC or DSTC) and then 0·2 ml. of NaOH solution (final pH of mixture, 12·4). Control tubes were set up with known amounts of aldose or ketose, and the mixtures incubated in a water bath at 37°. After a time specified below, the reaction was terminated and the pigment formed completely dissolved by adding 2·0 ml. of acidic solvent (acidic methanol with TTC; acidic propanol with DSTC). The colour is stable in the acidified solution at room temperature for at least 2 hr. The extinction was measured at 485 mμ with TTC and at 510 mμ with DSTC. Measurements were carried out in the Beckman DU spectrophotometer in cells of 1 cm. light-path. The final volume of reaction mixture was 3·0 ml.

Within wide ranges of sugar concentration and reaction time (see Experimental), the reduction of tetrazolium progresses linearly with time at a rate which varies directly as the sugar concentration. The following are suitable reaction times: 60 min. for aldose and 12 min. for ketose in the range 0·1–1·5 μmoles of sugar with TTC; 75 min. for aldose and 15 min. for ketose in the range 0·02–0·25 μmole of sugar with DSTC.

The sensitivity of the test is increased eightfold if the reaction time is doubled and the DSTC concentration increased fourfold.

## EXPERIMENTAL

At temperatures used in different procedures of sugar assay on the basis of reaction with tetrazolium (Fischer & Dörfel, 1954; Mattson & Jensen, 1950; Fairbridge, Willis & Booth, 1951) both inulobiose (1-R-fructose) and levanbiose (6-R-fructose) reduced TTC readily. At a relatively lower temperature (37°), however, a TTC reagent described by Cheronis (1956) manifested selective reactivity to 6-R-fructose and fructose as compared with 1-R-fructose, and to aldose as compared with 2-deoxyaldose (see below, 'Influence of carbohydrate structure'). Factors affecting the selective reactivity of this reagent were therefore examined more fully.

*Influence of* pH. The velocity of reaction was markedly increased with increase in pH, as has been previously noted by Cheronis (1956). At pH 11·4 (0·1 ml. of 0·2N-sodium hydroxide, 0·1 ml. of 0·2N-sodium tetraborate, 0·1 ml. of TTC solution and 0·7 ml. of sugar solution), for instance, the rate of reduction of TTC by glucose was not more than one-fiftieth of that at pH 12·4 (standard system). Tetrose (erythrose), trioses (glyceraldehyde, dihydroxyacetone) and glycolaldehyde, however, reduced TTC rapidly even at pH 11·4 [$E^{485\ m\mu}_{1\ cm.}$/μmole (in 3·0 ml. of reaction mixture)/10 min., 0·3, as compared with < 0·02 for glucose].

*Choice of oxidant.* Rate of production of pigment with DSTC was about five times that with TTC, both with ketose and aldose. Acidic methanol, a good solvent of TTC–formazan (Fischer & Dörfel, 1954), failed to dissolve the DSTC product readily. DSTC–formazan proved, however, to be readily soluble in acidic propanol.

*Influence of time and concentrations of reactants.* The course of the reduction was linear in time over a major part of the reaction. The initial reaction rate varied directly with the sugar amount over a wide range (Fig. 1) and affords a ready means of measuring sugar concentration (Fig. 2). As may be seen in Fig. 2, the limit yield in contrast with the rate of formation of formazan was constant in the designated range of carbohydrate amount (0·25–0·50 μmole). With smaller amounts of carbohydrate (< 0·2 μmole), the limit yield of formazan varied positively with the carbohydrate amount, but in a non-linear manner. Increase in the concentration of oxidant gave some increase in initial reaction rate and permitted enhancement of the sensitivity of the reaction without loss of selectivity (Fig. 3). The tested increase in oxidant concentration failed, however, to establish a condition in which the limit yield of formazan became a linear function of the sugar amount.

*Influence of temperature.* The reaction rates of glucose and fructose with TTC were about 1·3 times as much at 40° as at 37°. At 97° rates of the reaction were extremely high and difficult to measure. At this elevated temperature, moreover, selectivity as between levanbiose and inulobiose and as between glucose and 2-deoxyglucose was almost completely abolished.

*Influence of carbohydrate structure.* In the aldose series, changes at C-2 which impede 1:2-enediol isomerism resulted in marked depression of the reaction rate. The 2-deoxyaldoses tested, for example, were all almost non-reactive. A variety of alterations (e.g. epimerization, substitution of radicals in hydroxyl at C-4 and C-6, elongation or

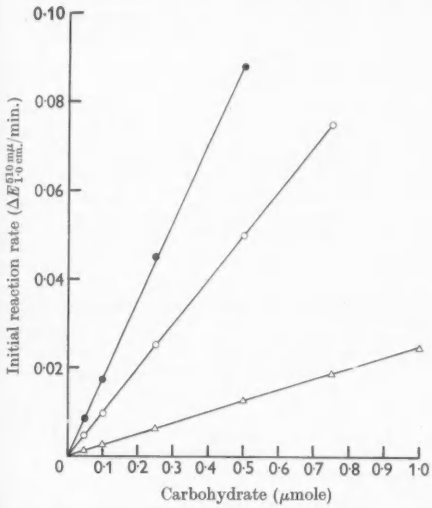


Fig. 1. Influence of concentration and type of reductant on initial rate of reaction. Standard reaction conditions were used, with DSTC as oxidant and with fructose (○), glucose (△) and levanbiose (●) as reductant. With TTC, as with DSTC, reaction rate was a linear function of the carbohydrate concentration, rates of inulobiose and levanbiose with TTC being as 1:20.

restricted abbreviation of carbon chain) effected in aldose at carbon sites other than C-2 failed to depress the reaction rate. An exception in this respect occurred, however, in two methylpentoses (L-rhamnose, L-fucose) but not in a third (D-thevetose). Substitution of $-CH_2 \cdot OH$ for $-H$ at C-1 in aldose, to yield ketose, resulted in a marked enhancement of reaction rate. Fructose reacted about five times as rapidly as glucose (cf. Fig.1). Abbreviation of the length of the carbon chain to afford triose (D-glyceraldehyde) likewise gave a marked enhancement of the reaction rate. The aldoses tested and their reactivities are listed in Table 1.

In the ketose series, substitution of glycosyl in the OH group at C-1 gave a marked depression in reaction rate. For example, equimolar amounts of inulobiose, inulotriose and higher members of this series reacted with TTC about one-twentieth as rapidly as levanbiose and about one-tenth as rapidly as fructose. This may be related to the suppressive effect of substitution at C-1 on 1:2-enediol isomerism. However, normal ketose reactivity characterized acetylmethylcarbinol. Substitution at C-6 in fructose with resulting restriction in the size of the oxygen ring gave a significant increase in the reaction rate observed both with TTC and DSTC (cf. levanbiose with fructose, Fig. 1). The size of the substituent on C-6 did not alter the rate of reaction appreciably, since equal

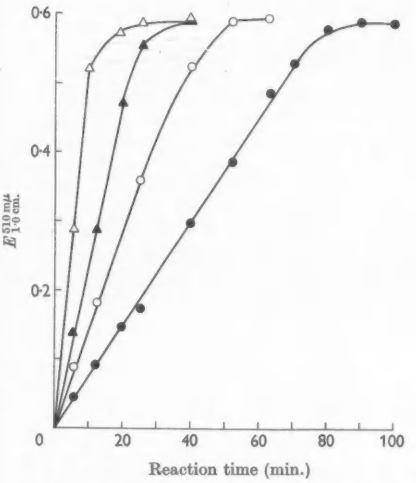


Fig. 2. Course of formazan production as a function of time. Standard reaction mixture with DSTC as oxidant. Fructose (μmole): 0·5 (△); 0·25 (▲). Glucose (μmole): 0·5 (○); 0·25 (●).

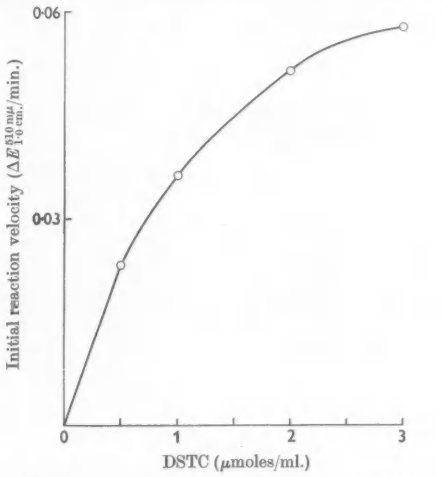


Fig. 3. Influence of oxidant concentration on initial rate of reaction. Standard reaction conditions were used, but at varying concentrations of DSTC with 0·25 μmole of glucose. In the tested range, the reaction was followed throughout its linear course.

Table 1. *Reaction of aldoses and related substances with triphenyltetrazolium chloride*

A standard reaction mixture (3·0 ml.) was used, with 60 min. incubation time. Sugars are of D-configuration except where otherwise specified.

| | | |
|---|---|---|
| | *Reactive compounds* ($E^{485\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, 0·4 ± 0·1) | |
| Monosaccharides | Unsubstituted sugars | Glycolaldehyde,* glyceraldehyde,* erythrose*<br>Ribose,† ribose 6-phosphate, xylose,† arabinose,†<br>L-rhamnose,‡ L-fucose‡<br>Glucose,† galactose,† mannose,† glucose 6-phosphate<br>D-*glycero*-L-*galacto*Heptose, D-*threo*-L-*galacto*-octose |
| | Uronic acids | Glucuronate,† galacturonate,† glucurono-δ-lactone |
| | Amino sugars | Glucosamine,† *N*-acetylglucosamine‡ |
| | *O*-Methyl-substituted | 6-Deoxy-3-*O*-methylglucose (thevetose), 3-*O*-methylglucose |
| Disaccharides | 3-Glycosylglycose<br>4-Glycosylglycose<br>6-Glycosylglycose | Nigerose, laminaribiose<br>Maltose,† cellobiose,† lactose<br>Isomaltose, gentiobiose, melibiose,† 6-fructosylglucose |
| | *Relatively inert compounds* ($E^{485\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, ⩽0·01) | |
| Monosaccharides | 2-Deoxyaldoses | 2-Deoxyribose,§ 2-deoxyglucose,§ 2-deoxyallose,<br>2-deoxygalactose,§ 2:6-dideoxyallose (digitoxose) |
| | 2-*O*-Methylaldoses | 2-*O*-Methylglucose, 2:3-di-*O*-methylglucose,<br>2:3-di-*O*-methylxylose |
| Disaccharides | 2-Glycosylglycoses | Sophorose, 2-fructosylglycose, kojibiose,<br>2-β-xylosyl-L-arabinose |
| | 1-Glycosylglycoses | Sucrose, trehalose |
| Other compounds | — | Methyl α-glucoside, α-glucose 1-phosphate, formaldehyde,<br>acetaldehyde, glyoxalate, pyruvate, oxaloacetate,<br>oxoglutarate |

* Reaction conducted at pH 11·4. † With DSTC this sugar gave $E^{510\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, 2·0 ± 0·4.
‡ Compound showed relatively low activity ($E^{485\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, 0·04–0·08).
§ With DSTC this sugar gave $E^{510\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, ⩽0·01.

Table 2. *Reaction of ketoses with triphenyltetrazolium chloride*

A standard reaction mixture (3·0 ml.) was used, with 12 min. incubation time. Sugars are of D-configuration except where otherwise specified.

| | | |
|---|---|---|
| | *Reactive compounds* ($E^{485\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, 0·4 ± 0·1) | |
| Monosaccharides | Unsubstituted sugars | Dihydroxyacetone*<br>Xylulose<br>Fructose,† tagatose,† L-sorbose,† fructose<br>6-phosphate<br>Glucoheptulose, sedoheptulose† |
| | Oxogluconate | 2-Oxogluconate, 5-oxogluconate |
| | Other compounds | Acetylmethylcarbinol |
| Di- and oligo-saccharides | — | Turanose<br>Maltulose<br>Isomaltulose, levanbiose, oligolevans (*n*, 3–8) |
| | *Relatively inert compounds* ($E^{485\,m\mu}_{1\cdot0\,cm.}$/mole, ⩽0·05) | |
| Compounds of type 1-R-fructose | — | 1-Glucosylfructose, inulobiose,‡ oligoinulins<br>(*n*, 3–8),‡ fructose 1:6-diphosphate, A-series of<br>oligolevans (Zelikson & Hestrin, 1961)‡ |
| Other compounds | — | Acetylacetone |

* Reaction conducted at pH 11·4.
† With DSTC this sugar gave $E^{510\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, 2·0 ± 0·4. ‡ $E^{510\,m\mu}_{1\cdot0\,cm.}$/$\mu$mole, 0·045.

yields of colour per mole were found with levanbiose, levantriose and higher members of this series. A variety of alterations effected at C-3, C-4 or C-5 (e.g. introduction of substituents, epimerization, elongation or restricted abbreviation of carbon chain) did not markedly affect the reaction rate. A compound (2-oxogluconate) in which the keto group is vicinal both to a secondary carbinol and to a carboxyl group reacted with about the same rapidity as fructose itself. Abbreviation of the carbon chain to afford triose (dihydroxyacetone) gave a marked enhancement in the rate of reaction. The classes of ketoses tested and their reactivities are listed in Table 2.

*Interfering substances.* Several alcohols (methanol, ethanol, propan-2-ol) markedly augmented the rate at which formazan was formed from TTC by the action of a variety of ketoses. The selectivity of the essay as between levanbiose and inulobiose was almost completely abolished by small amounts of such solvents, e.g. by 0·2 ml. of methanol in 3·0 ml. of standard reaction mixture. When TTC was used as a spray on paper, the selectivity of the reagent as between compounds of the type of 1-R- and 6-R-fructose was likewise almost abolished in the presence of several common alcohols (butan-1-ol, propan-1-ol, propan-2-ol, ethanol, methanol).

## DISCUSSION

The method presented above permits the selective determination of 6-R-fructose or free fructose in the presence of an equal amount of 1-R-fructose and that of aldose in the presence of an at least thirtyfold quantity of 2-deoxyaldose within limits of error less than 5 %. With DSTC as oxidant, under conditions of prolonged reaction and elevated concentration of oxidant, as little as 5 $\mu$m-moles of sugar can be determined by means of this technique.

Although the proposed method is simple, rapid and highly selective, its reliability as an analytical procedure in application to biological mixtures suffers from the circumstance that the measurement of sugar concentration is based on rate of formation rather than on limit yield of formazan.

## SUMMARY

1. Tetrazolium derivatives in aqueous solution at pH 12·4 are readily reduced at 37° by sugars manifesting enediol isomerism, but not by sugars in which such isomerism is impeded by presence of a substituent in a carbinol group vicinal to the carbonyl group.

2. A method is presented for the determination of 6-R-fructose and fructose in the presence of 1-R-fructose, and of aldose in the presence of 2-deoxyaldose by means of reaction with tetrazolium derivatives. Conditions are defined in which the rate of the reduction is a linear function of the sugar concentration over a wide concentration range.

3. The use of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium in this reaction permits smaller amounts of sugar to be determined than was possible with the use of 2:3:5-triphenyltetrazolium.

4. The selectivity of reaction as between 6-R- and 1-R-fructose is abolished if certain alcohols at high concentration are present in the reaction mixture during formazan production.

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# Condensed Tannins

## 10. ISOLATION OF (−)-BUTIN AND BUTEIN FROM WATTLE HEARTWOODS

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The present study shows that in addition to the extractives described by Roux & Paulus (1960, 1961*a*) and Roux, Maihs & Paulus (1961) wattle heartwoods contain very low proportions of the isomeric compounds, (−)-butin [(−)-7:3′:4′-trihydroxyflavanone] and butein (2:4-dihydroxyphenyl-3:4-dihydroxystyryl ketone).

### EXPERIMENTAL AND RESULTS

All melting points were obtained in soda glass and are uncorrected. Mixed melting points are on molecular mixtures of compounds (Roux & Maihs, 1960). Analyses of C, H, methoxyl and acetyl are by Weiler and Strauss, Oxford. Infrared comparisons are by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. Two-way chromatograms were developed in water-saturated butan-2-ol followed by 2% (v/v) acetic acid.

*Separation of (−)-butin from black-wattle heartwood.* The contents of tubes 146–160 from three 160-transfer Craig countercurrent partition separations, water–butan-2-ol–light petroleum (5:3:2) and an enriched flavonoid fraction being used for separations (Roux & Paulus, 1961*a*), were united. The organic phase was separated and the aqueous layer extracted with ethyl acetate. The combined organic phase and ethyl acetate extracts were reduced to 100 ml. and applied as a band (5 ml./sheet) to 20 sheets ($22\frac{1}{2}$ in. × $18\frac{1}{2}$ in.) of Whatman no. 3 chromatographic paper. The chromatograms were developed by upward migration in 2% acetic acid. A reducing band $R_F$ 0·25 was located by ammoniacal silver nitrate on a test strip. Bands were cut and eluted with 70% ethanol. The eluents were evaporated to dryness under reduced pressure in a rotary evaporator, and the solids crystallized (charcoal) from water containing a little ethanol. Colourless flat plates (100 mg.) m.p. 223–224° were obtained from the methanol extract (360 g.) of the heartwood (9·9 kg.) (Roux & Paulus, 1961*a*) [Found: C, 58·1; H, 5·0; loss at 120° *in vacuo* (2 hr.), 11·4. $C_{15}H_{12}O_5$, $2H_2O$ requires: C, 58·4; H, 5·2; $H_2O$, 11·7%]. The anhydrous compound had m.p. 223–224°, not depressed by admixture with (±)-butin m.p. 223–224°; $[\alpha]_D^{20}$ − 18·6±0·8° in methanol (*c*, 0·6) (Found: C, 66·4; H, 4·5. $C_{15}H_{12}O_5$ requires: C, 66·2; H, 4·4%). $\lambda_{max.}$ 234, 280 and 311 mμ in ethanol, and 254 and 336 mμ in a 0·1% soln. of sodium ethoxide in ethanol. The infrared-absorption spectrum of the compound (KBr disk) had strong bands at 3450 (OH), 1650 (pyrone C:O), 1600, 1530 and 1465 (aromatic C:C), 1280 (aromatic C:O) and 1120 (aryl–alkyl ether), but with low absorption over the range 1100–1000 cm.$^{-1}$ compared with the strong peak at 1015 cm.$^{-1}$ of fustin [secondary aliphatic OH (?)]. The infrared-absorption and ultraviolet-absorption (cf. Harborne & Geissman, 1956) spectra and optical rotation suggested that the substance was (−)-butin. It gave resorcinol, β-resorcylic acid and protocatechuic acid on microfusion with KOH under temperature-controlled anhydrous conditions (Roux, 1958). On chromatograms the colours given with spray reagents were yellow (bis-diazotized benzidine), green–grey (ferric alum) and yellow (under ultraviolet light after treatment with ammonia).

*O-Trimethyl-(−)-butin.* (−)-Butin (70 mg.) in methanol (20 ml.) was methylated at −10° for 12 hr. with excess of dry ethereal diazomethane. *O*-Trimethyl-(−)-butin (40 mg.) was recrystallized from ethanol as colourless plates, m.p. 114–115°; $[\alpha]_D^{20}$ − 16·3±0·2° in tetrachloroethane (*c*, 0·9) (Found: C, 68·9; H, 5·8; OMe, 30·3. $C_{18}H_{18}O_5$ requires: C, 68·8; H, 5·8; OMe, 29·6%).

*(−)-Butin from golden-wattle heartwood.* (−)-Butin (100 mg.) was isolated from 250 g. of methanol extract of golden-wattle heartwood (5·1 kg.) as described above (cf. Roux *et al.* 1961); m.p. 223–224°; $[\alpha]_D^{20}$ − 18·7±0·8° in methanol (*c*, 0·5) and −10·1±0·8° in acetone–water (1:1, v/v) (*c*, 0·7). The ultraviolet- and infrared-absorption curves were the same as those of (−)-butin from black wattle, and a mixed m.p. of these compounds showed no depression.

*Triacetyl-(−)-butin.* (−)-Butin (70 mg.) from golden-wattle heartwood was acetylated with 0·3 ml. of pyridine and 0·35 ml. of acetic anhydride at room temperature for 2 hr. The product obtained by pouring into water crystallized from ethanol in white clusters (37 mg.) and had m.p. 121–122°. Recrystallization gave white shining platelets m.p. 121–122°; $[\alpha]_D^{20}$ − 12·5±0·3° in tetrachloroethane (*c*, 0·5) (Found: C, 63·3; H, 4·6. $C_{21}H_{18}O_8$ requires C, 63·2; H, 4·7%).

The presence of butin in the heartwoods of green (*Acacia decurrens*) and silver (*Acacia dealbata*) wattles was shown by paper chromatography.

*Butin from commercial quebracho extract.* Contents of tubes 145–160 of a Craig separation of a flavonoid-enriched fraction (320 g.) of commercial quebracho extract (Roux & Paulus, 1961*b*) were combined, and the components separated on paper sheets with 2% (v/v) acetic acid as above. A band corresponding to butin was cut and eluted but failed to crystallize under identical conditions. Paper chromatograms showed that the fraction was complex but that butin was the predominant component. Running the fraction again on two sheets of Whatman no. 3 paper in 2% acetic acid gave 1·5 mg. of plates from ethanol–water, m.p. 220–221° not depressed by admixture with (±)-butin, m.p. 220–222°. $\lambda_{max.}$ 234, 280 and 310 mμ in ethanol, and

254 and 336 mμ in a 0·1% soln. of sodium ethoxide in ethanol. Colour reactions on paper chromatograms were identical with those of (−)-butin from black-wattle wood.

*Synthesis of* (±)-*butin.* Alkaline isomerization of butein (Saiyad, Nadkarni & Wheeler, 1937) gave a 32% yield of (±)-butin, m.p. 223–224°, whereas acid isomerization (Geissman & Clinton, 1946) gave 7% of (±)-butin, m.p. 225–226°. Reichel, Burkart & Muller (1942) report a 37% yield of (±)-butin from butein by acid isomerization. (±)-Butin was separated from unchanged butein by chromatography on Whatman no. 3 paper with 2% acetic acid. Ultraviolet-absorption (range 230–340 mμ) and infrared-absorption (range 2·5–15 μ) curves of synthetic and natural butins were superimposable. Their $R_F$ values were identical in 2% acetic acid (0·22) and in water-saturated butan-2-ol (0·92), with (+)-fustin (0·37, 0·82 respectively) as reference substance.

*O-Trimethyl-*(±)-*butin.* (±)-Butin was methylated as was (−)-butin and gave a product with m.p. 118–119°. The mixed m.p. with *O*-trimethyl-(−)-butin (m.p. 114–115°) was m.p. 118°. The infrared-absorption curves of these substances were identical over the range 2·5–15 mμ.

*Triacetyl-*(±)-*butin.* (±)-Butin was acetylated as was (−)-butin. The product had m.p. 124–126°. The mixed m.p. with triacetyl-(−)-butin (m.p. 121–122°) was 123–125°.

*Isolation of butein from golden-wattle heartwood.* Those components in tubes 146–160 from the Craig counter-current separation which remain at the origin during subsequent separation of (−)-butin on paper sheets, were cut out and eluted with 90% ethanol. Fisetin and a yellow substance were shown present in the eluents by paper chromatography with butan-1-ol–acetic acid–water (6:1:2, by vol.). The solids (2·5 g.) were applied to Whatman no. 3 sheets, which had been washed previously with water for 24 hr., and the chromatograms developed in the above-named solvent system. A yellow band running immediately in front of fisetin was cut and eluted with 70% ethanol. The yellow solids were recrystallized from ethanol containing a little water to yield bright-yellow crystals, m.p. 211° (8 mg. from 5·1 kg. of heartwood). The infrared-absorption curves of the substance and of synthetic butein (m.p. 212°) were superimposable. A mixed m.p. of these substances showed no depression (211°).

Butein was also found in black-wattle (*Acacia mearnsii*), silver-wattle (*A. dealbata*) and green-wattle (*A. decurrens*) heartwood extracts.

## DISCUSSION

(−)-Butin and the corresponding chalkone, butein, have been shown to occur as minor components in the heartwood extracts of *Acacia mearnsii* and *A. pycnantha* by isolation and direct comparison with synthetic compounds. These compounds add to the already complex pattern of inter-related heartwood components, all of which are based on a combination of resorcinol and catechol nuclei (cf. Roux & Paulus, 1960, 1961*a*).

Optically active butin has hitherto not been isolated from natural sources, and the absolute configuration of natural and synthetic butins has not been established. (−)-Butin [(−)-7:3′:4′-trihydroxyflavanone] from wattle heartwoods ($[\alpha]_D$ −18·7°) is likely to have the same (2S) configuration as (−)-liquiritigenin [(−)-7:4′-dihydroxyflavanone] from *Glycyrrhiza glabra* ($[\alpha]_D$ −36°) (Arakawa & Nakazaki, 1960*a*, *b*). (−)-Butin is therefore likely to have the same configuration at the asymmetric carbon atom 2 as the associated and related compounds (+)-fustin, (−)-fisetinidol and (+)-7:3′:4′-trihydroxyflavan-3:4-diol present in wattle heartwoods (Roux & Paulus, 1960, 1961*a*; Weinges, 1958, 1959).

## SUMMARY

1. (−)-Butin and butein have been isolated from black- and golden-wattle heartwoods.

2. Traces of butin exist in quebracho heartwoods.

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# Aconitase Levels in the Leaves of Iron-Deficient Mustard Plants (*Sinapis alba*)

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There have been a number of reports that aconitase in animal tissues is affected by disturbances of iron or ascorbic acid metabolism. Some studies have been made with guinea pigs (Takeda & Hara, 1955; Banerjee & Singh, 1960) but with discordant results. Beutler (1959) has reported that the aconitase level in kidney homogenates from rats fed on an iron-deficient diet was only about half that of controls receiving iron supplements; the activity of the homogenates could not be raised to the normal level by incubation with ascorbate and ferrous sulphate.

We have reported a similar depression of aconitase activity in the leaves of mustard plants (Bacon, DeKock & Palmer, 1959). The present paper describes these experiments in detail and provides further evidence for a specific effect of iron-deficiency on aconitase in this species.

## METHODS AND MATERIALS

*Growth of plants.* A description of most aspects of the growing of the mustard plants has already been given (see DeKock, Hall & MacDonald, 1960*b*). The seeds were germinated in sand and transferred after 1 week to solution culture, and the seedlings in each crock thinned to 15. The first samples for analysis were taken when flower buds were visible, usually 3 weeks after sowing. The plants were grown in a greenhouse, and during the winter (September to April) received extra illumination (about 400 foot-candles) from fluorescent tubes suspended about 30 cm. above the crocks. These tubes were switched on from 5 a.m. to 9 p.m. (G.M.T.) each day and provided local heating as well as light. The ambient temperature of the greenhouse was maintained between 5° and 20° during cold weather, and the plants were shielded from direct sunlight during sunny weather.

*Enzyme assays.* Aconitase and fumarase were measured as described by Bacon, Palmer & DeKock (1961), and are expressed respectively as μmoles of *cis*-aconitate or fumarate formed/g. fresh wt./hr.

Malic dehydrogenase was measured in the same homogenate by a modification of the method applied by Price & Thimann (1954) to particulate fractions from disintegrated leaf tissue. Before use the homogenate was titrated with 0·8 mM-2:5-dichlorophenol-indophenol until a slight blue colour persisted; in this way the interference of ascorbic acid and other reducing substances was eliminated. The equivalent of 0·05 ml. of original homogenate was added to two test tubes each containing 0·5 ml. of freshly prepared 0·2 M-KCN, 0·5 ml. containing 1 mg. of diphosphopyridine nucleotide (DPN), 0·25 ml. of 0·8 mM-2:5-dichlorophenol-indophenol and 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol(tris)–HCl buffer (pH 7·5) to a final volume of 4 ml. Each tube was read against water in an EEL portable colorimeter, with a no. 625 (green) filter; 0·25 ml. of 0·3 M-sodium L-malate was added to one and buffer to the other and readings were repeated every 2 min. for 12 min. No change in absorption occurred in the control; decolorization of the test solutions was approximately linear with time and the fall in absorption between 2 min. and 10 min. was used to calculate the enzyme activity, which is expressed as μmoles of malic acid oxidized/g. fresh wt. of leaf/hr.

*Other estimations.* Chlorophyll was extracted with acetone from fresh leaf samples and measured spectrophotometrically (cf. Arnon, 1949).

Ascorbic acid was measured in metaphosphoric acid extracts by titration with 2:5-dichlorophenol-indophenol (Roe, 1954).

Dry matter was measured by drying to constant weight at 80°.

Kjeldahl nitrogen was measured in fresh material by a micromethod.

*Materials.* The substrates described by Bacon *et al.* (1961) were used. Potassium dihydrogen (+)-*iso*citrate, prepared from the leaves of *Sedum prealtum* by the method of Vickery & Wilson (1958), was used in some aconitase assays in place of sodium (±)-*iso*citrate prepared from a commercial sample of the lactone, but with no effect upon the values obtained (cf. Pierpoint, 1960).

## RESULTS

Plants were grown in the presence of 0·2, 1·0 and 5·0 p.p.m. of iron, in most cases supplied as the ferric salt of ethylenediaminetetra-acetic acid (ferric EDTA). Those receiving only 0·2 p.p.m. were always chlorotic and often considerably stunted; no obvious differences were seen between plants receiving 1·0 and 5·0 p.p.m. of iron, but measurements of height and fresh weight, and scoring for chlorosis have shown that there is some response to the higher concentration (cf. DeKock *et al.* 1960*b*). When iron was supplied as the ferric salt of *NN'*-ethylenebis-[2-(*o*-hydroxyphenyl)glycine] (Chel 138; Geigy Chemical Co. Ltd., Manchester) growth was good and chlorosis slight at the 0·2 p.p.m. concentration. DeKock *et al.* (1960*b*) found no significant effect of iron concentration on

Table 1. *Aconitase activities of leaves from plants receiving various concentrations of iron, potassium, calcium, phosphate and nitrate*

Seeds were sown on 18 July 1958 and transferred to nutrient solution in 5 l. glazed crocks on 23 July 1958. The various treatments were arranged in a $4 \times 3 \times 2$ factorial design, each treatment being repeated twice and the treatments randomized in two blocks of 24. The concentrations of the various nutrients were as follows: Fe 1, 2, 3,: 0·2, 1·0, and 5·0 p.p.m. of iron as ferric EDTA; K/Ca 1, 2, 3, 4, represent the following ratios of potassium to calcium in a total of 13 m-equiv./l.: 1/12, 3/10, 6/7, 10/3; P/N 1, 2, represent respectively ratios of phosphate to nitrate of 1/11 and 2/10 in a total of 12 m-equiv./l. Samples (0·5 g.; 8–10 leaves) were taken from each crock for measurement of aconitase activity. Three analyses were made on 12–13 August and the remainder between 22 August and 1 September.

| | Fe 1 | Fe 2 | Fe 3 | P/N 1 | P/N 2 | Mean | |
|---|---|---|---|---|---|---|---|
| K/Ca 1 | 7 | 19 | 45 | 26 | 22 | 24 | |
| K/Ca 2 | 10 | 21 | 45 | 33 | 18 | 25 | S.E.M. ±2·2 |
| K/Ca 3 | 9 | 18 | 43 | 23 | 24 | 23 | |
| K/Ca 4 | 10 | 19 | 44 | 20 | 28 | 24 | |
| P/N 1 | 10 | 17 | 49 | | | 25 | S.E.M. ±1·5 |
| P/N 2 | 8 | 22 | 40 | | | 23 | |
| Mean | 9 | 19 | 44 | | | | |
| | | S.E.M. ±1·9 | | | | | |

S.E.M. of 4 in K/Ca × Fe table ±3·8
S.E.M. of 6 in K/Ca × P/N table ±3·1
S.E.M. of 8 in P/N × Fe table ±2·7

Table 2. *Repetition of experiment of Table* 1

Seeds were sown on 17 March 1959, transferred to crocks on 25 March 1959 and sampled from 9 to 17 April 1959. All leaf samples were taken from position 1, the first true leaf.

| | Fe 1 | Fe 2 | Fe 3 | P/N 1 | P/N 2 | Mean | |
|---|---|---|---|---|---|---|---|
| K/Ca 1 | 10 | 22 | 5 | 11 | 14 | 13 | |
| K/Ca 2 | 8 | 22 | 6 | 10 | 14 | 12 | S.E.M. ±1·6 |
| K/Ca 3 | 9 | 18 | 2 | 11 | 9 | 10 | |
| K/Ca 4 | 8 | 16 | 18 | 13 | 14 | 14 | |
| P/N 1 | 9 | 19 | 6 | | | 11 | S.E.M. ±1·1 |
| P/N 2 | 8 | 20 | 10 | | | 13 | |
| Mean | 9 | 20 | 8 | | | | |
| | | S.E.M. ±1·3 | | | | | |

S.E.M. of 4 in K/Ca × Fe table ±2·7
S.E.M. of 6 in K/Ca × P/N table ±2·2
S.E.M. of 8 in P/N × Fe table ±1·9

the height or fresh weight of such plants. The concentrations of iron used were incorrectly stated (as 0·1, 0·5, 2·5 p.p.m.) in a preliminary communication (Bacon *et al.* 1959) and also by DeKock, Commisiong, Farmer & Inkson (1960*a*). Some of the plants used in the present experiments were in fact grown at these lower concentrations of iron (see Tables 4 and 5), and these received part of their nitrogen in the form of ammonium salts, but the majority of plants used are directly comparable with those analysed for mineral constituents by DeKock *et al.* (1960*b*), and for peroxidase, catalase, haematin pigments and chlorophyll by DeKock *et al.* (1960*a*).

*Effect of concentration of iron on actonitase activity*

Table 1 shows the results of growing mustard plants in solutions in which the concentrations of phosphorus, potassium and calcium, as well as of iron, were varied. A factorial arrangement with duplication of treatments provided 48 crocks for analysis. The majority of the measurements were made during a period of 12 days at the end of August 1958. Table 2 summarizes the results of a similar experiment carried out in April 1959. The 12 possible combinations of iron concentrations and potassium/calcium (K/Ca) ratios are represented by the figures (each the mean of four crocks) in the top left-hand corner of each table; the six combinations of iron concentration with phosphate/nitrate (P/N) ratios are given below them, and the eight combinations of K/Ca ratio with P/N ratio on their right. In the margins are the mean values for all crocks receiving the same concentration of a particular nutrient: thus there were 12 crocks

Table 3. *Effect of leaf position on aconitase and malic dehydrogenase activities*

Seeds were sown on 29 May 1959 and transferred to 24 crocks on 4 June 1959. Iron concentration was the same as in Tables 1 and 2; in one block of 12 crocks it was given as ferric EDTA, in the other as ferric Chel 138. In all crocks K/Ca was 3/10 and P/N 2/10. Samples (0·5 g.; 8–10 leaves) from position 2 were analysed on 19–24th June and similar samples from position 5 of the same plants on 24–29th June. Aconitase and malic-dehydrogenase activities were measured on the same homogenate and are expressed respectively as μmoles of *cis*-aconitate formed or malate oxidized/g. fresh wt./hr.

| | Chelate | Leaf position | Concn. of iron (p.p.m.) 0·2 | 1·0 | 5·0 |
|---|---|---|---|---|---|
| Aconitase | EDTA | 2 | 14 | 21 | 8 |
| | | 5 | 17 | 39 | 36 |
| | Chel 138 | 2 | 18 | 30 | 33 |
| | | 5 | 18 | 44 | 52 |
| Malic dehydrogenase | EDTA | 2 | 35 | 38 | 40 |
| | | 5 | 47 | 56 | 56 |
| | Chel 138 | 2 | 41 | 46 | 45 |
| | | 5 | 62 | 56 | 67 |

receiving each K/Ca ratio, 24 each P/N ratio, and 16 each iron concentration.

In each case iron had a marked effect on the aconitase activity, 1·0 p.p.m. of iron giving approximately twice the activity with 0·2 p.p.m. This effect was confirmed in numerous later experiments. The effects of alterations in the other constituents were slight or inconsistent, and for many subsequent experiments plants were grown with variations of the iron concentration only, the K/Ca ratio being fixed at 3/10 and the P/N ratio at 2/10.

With 5·0 p.p.m. of iron there was a striking difference between the two experiments, the aconitase activity being higher than at 1·0 p.p.m. in one (Table 1) and lower in the other (Table 2). Two differences in experimental conditions were considered: (*a*) in the first experiment only natural illumination was used, while in the second intense artificial illumination supplemented the rather weak natural light; (*b*) a different leaf was chosen for analysis in the second experiment. Since the difference in illumination could not easily be reproduced attention was paid to leaf position.

The mustard plants grown with an adequate supply of iron under our conditions have an unbranched stem with eight or nine leaves before the terminal inflorescence. In the first experiment the third leaf down from the inflorescence, i.e. leaf number 6 or 7, was taken from the plants receiving 1·0 and 5·0 p.p.m. of iron, but, because they failed to develop more than two or three leaves, leaf 1 or 2 had to be used from the 0·2 p.p.m. plants. For the second experiment leaf 1 was taken from all plants. Table 3 shows results of an experiment in which enzyme activities of leaves 2 and 5 were compared.

With iron supplied as ferric EDTA the aconitase activity of leaf 2 was much less with 5·0 p.p.m. of iron than with 1·0 p.p.m. (cf. Table 2), but that of leaf 5 was only slightly less. In both leaves the activity with 1 p.p.m. was greater than with 0·2 p.p.m. Table 3 also gives the results of an experiment conducted simultaneously on an adjoining bench, in which iron was given as ferric Chel 138. Because the crocks were not randomized with respect to chelate this difference may not be wholly responsible for the large differences in absolute activities which were found, particularly at the higher concentrations of iron. The general pattern of response to iron was not significantly

Table 4. *Simultaneous measurements of aconitase action on* isocitrate, cis-*aconitate and citrate*

Seeds were sown on 7 April 1960 and transferred to 12 crocks on 15 April. In the nutrient solution some nitrogen was supplied as ammonium salts (5 m-equiv./l.), the remainder as nitrate (10 m-equiv./l.); phosphate, potassium, and calcium were present at 2, 3, and 5 m-equiv./l. respectively. The crocks were randomized as one block, each concentration of iron (ferric EDTA: 0·1, 0·5, 2·5, p.p.m.) being repeated four times. Samples (0·5 g.; 8–10 leaves; position 2) were taken on 27–29 April. Each was homogenized and tested in the standard assay system with 0·05 M-(±)-*iso*citrate, 0·25 mM-*cis*-aconitate or 0·1 M-citrate as substrate; the assays were carried out simultaneously at room temperature (26–27°). All activities are expressed as μmoles of *cis*-aconitate formed or disappearing/g. fresh wt./hr.

| Substrate | Concn. of iron (p.p.m.) | Aconitase activity: Individual measurements | Mean |
|---|---|---|---|
| *iso*Citrate | 0·1 | 8, 6, 7, 5 | 6 |
| | 0·5 | 17, 16, 13, 23 | 17 |
| | 2·5 | 29, 37, 27, 43 | 34 |
| *cis*-Aconitate | 0·1 | 8, 5, 4, 4 | 5 |
| | 0·5 | 13, 13, 11, 15 | 13 |
| | 2·5 | 18, 24, 21, 24 | 22 |
| Citrate | 0·1 | 2, 5, 6, 4 | 4 |
| | 0·5 | 6, 13, 10, 11 | 10 |
| | 2·5 | 18, 16, 13, 22 | 17 |

Table 5. *Effect of iron concentration on dry-matter content of leaves*

In experiment (*a*) sufficient leaves from position 3 were taken from each crock to give a sample weight of 1·0–1·2 g.; each figure represents one crock. In the other two experiments all the leaves from position 1 were taken from each crock, the sample weight varying from 0·5 to 1·1 g.; each figure is the average of the results from two crocks. In (*a*) the K/Ca ratios were as in Table 1, and P/N was 1/11; in (*b*) and (*c*) the nutrient solution was that described in Table 4. In each experiment the dry matter is expressed as per cent fresh weight.

| | 0·2 p.p.m. of iron | | | 1·0 p.p.m. of iron | | |
|---|---|---|---|---|---|---|
| | K/Ca 1 | K/Ca 2 | K/Ca 3 | K/Ca 1 | K/Ca 2 | K/Ca 3 |
| (*a*) | 11·7 | 10·3 | 10·3 | 6·5 | 9·6 | 8·3 |
| | | Mean 10·5 | | | Mean 8·1 | |
| | | Concn. of iron (p.p.m.) | 0·1 | 0·5 | 2·5 | |
| (*b*) | | | 8·3 | 8·5 | 9·1 | |
| (*c*) | | | 9·7 | 8·9 | 10·2 | |

affected by the difference in chelates, but when Chel 138 was used the aconitase activity in both leaves was greater with 5·0 p.p.m. of iron than with 1·0 p.p.m., suggesting that the form in which iron is supplied may also have an influence on the response to the higher concentration.

There was a variable response to 5·0 p.p.m. of iron in subsequent experiments in which leaf 1 or 2 was used (cf. Table 6). Low values were usually characterized by extreme variability: thus the individual values for the assays (at Fe 3) in Table 2 were: 20,* 5, 2, 12, 2, 18,* 0, 0, 9, 0, 10,* 10, 0, 0, 14, 22,* and for Fe 3-EDTA, leaf 2, in Table 3: 0, 0, 4, 27. The effect of the highest K/Ca ratio in Table 2 (values marked with *) in supporting a relatively high aconitase activity is not at all apparent in Table 1. In Table 6 (see below) the opposite effect is seen, the activity with 5·0 p.p.m. of iron decreasing with increasing K/Ca ratio.

The effect of iron concentration was similar when aconitase was measured with *cis*-aconitate or citrate instead of *iso*citrate as substrate. For example, Table 4 shows the results of simultaneous spectrophotometric measurements with all three substrates. A statistical examination was made of the differences between the logarithms of these measurements; it showed that the ratios of activity at 0·1 p.p.m. to that at 0·5 p.p.m. were indistinguishable for the three substrates, and so also were the ratios between activities at 0·5 and 2·5 p.p.m. These results support the view that essentially the same enzyme system is being measured in iron-deficient and normal plants. (In this experiment, carried out under winter conditions in April 1960, with leaf 2, the concentrations of iron were halved, and some nitrogen was supplied in the form of ammonium ions. It will be noticed that the enzyme activity at 2·5 p.p.m. was twice that at 0·5 p.p.m.)

*Other changes with concentration of iron*

The effect of iron concentration was further investigated to see whether it could be explained by changes in the gross composition of the leaf, or by a general depression of enzymic activity. Measurements on several batches of plants showed only small differences in dry matter (as % fresh weights) between leaves taken from plants receiving different concentrations of iron (Table 5). In one case the Kjeldahl nitrogen was also measured, giving values of 0·98, 0·90 and 0·83 g. of nitrogen/100 g. fresh wt. for 0·1, 0·5 and 2·5 p.p.m. of iron respectively. There was no consistent effect of iron on the ascorbic acid content of the leaves.

Malic-dehydrogenase and fumarase activities were measured simultaneously with aconitase in a few experiments. Table 3 includes measurements of malic dehydrogenase, which show a small but significant increase with iron concentration. As with aconitase, the activity of leaf 5 was throughout greater than that of leaf 2. The results of some simultaneous estimations of fumarase and aconitase are given in Table 6. Fumarase was significantly less at 1·0 p.p.m. than at 0·2 or 5·0 p.p.m.; a similar tendency was found in other experiments. Table 6 also shows an effect of K/Ca ratio on both enzyme activities at the 5·0 p.p.m. level.

*Changes during recovery from iron deficiency*

If the chlorotic leaves of iron-deficient plants are painted with solutions containing iron they slowly turn green, and their aconitase activity increases. However, this type of curative treatment is not a very satisfactory subject for study, because the leaves often show areas of 'scorching'. Two other types of experiment were therefore carried out.

*Feeding of iron to detached leaves.* Leaves from iron-deficient plants (0·1 p.p.m.) were detached from the plants and their petioles immersed in a nutrient solution identical with that used to grow the plant except for the omission of iron. These leaves served as controls. Others were placed in nutrient solution containing iron salts or chelates. If the leaves were kept under the same conditions of temperature and illumination as the parent

Table 6. *Effect of various concentrations of iron, potassium and calcium on the fumarase and aconitase activities of leaves*

Seeds were sown on 23 April 1960 and transferred to 24 crocks on 29 April. Levels of iron, potassium and calcium were as in Table 1; phosphate to nitrate 2/10 in all. The treatments were arranged in a $4 \times 3$ factorial design, each treatment being repeated twice, and the treatments were randomized in one block of 24. Samples (0·5 g.; 8–10 leaves; position 1) were analysed from 16 to 19 May 1960.

| Aconitase | K/Ca 1 | K/Ca 2 | K/Ca 3 | K/Ca 4 | Mean |
|---|---|---|---|---|---|
| Fe 1 | 4 | 6 | 8 | 12 | 8 |
| Fe 2 | 18 | 12 | 13 | 26 | 17 } S.E.M. ±2·6 |
| Fe 3 | 24 | 25 | 16 | 12 | 20 |
| Mean | 15 | 14 | 12 | 17 | |

S.E.M. ±3·0
S.E.M. of 2 in body of table ±5·2

| Fumarase | K/Ca 1 | K/Ca 2 | K/Ca 3 | K/Ca 4 | Mean |
|---|---|---|---|---|---|
| Fe 1 | 28 | 42 | 29 | 30 | 32 |
| Fe 2 | 24 | 19 | 12 | 26 | 20 } S.E.M. ±2·5 |
| Fe 3 | 33 | 42 | 22 | 16 | 28 |
| Mean | 28 | 34 | 21 | 24 | |

S.E.M. ±2·9
S.E.M. of 2 in body of table ±5·0

Table 7. *Effects of feeding iron to leaves detached from iron-deficient plants*

Plants were grown in solution containing 0·1 p.p.m. of iron as ferric EDTA and other nutrients as described in Table 4. Leaves were detached and a sample was assayed for aconitase. The petioles of the remainder were placed in culture solution, with or without iron supplements. The controls were assayed at the end of the experiment; the others at intervals. In some experiments chlorophyll was also estimated; the results are given as aconitase activities and (in parentheses) as μmoles of chlorophyll/g. fresh wt. For experiment (*a*) leaves from positions 1 and 2 were used; for the others from position 1 only. — Signifies that no measurement was made.

| Expt. | Form and concn. of iron | Time of treatment (days) 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| (*a*) | None | 10 | — | — | 9 | — |
| | Chel 138; 10 p.p.m. | — | 17 | 23 | 42 | — |
| (*b*) | None | 7 (0·63) | 9 (0·73) | — | 7 | — |
| | Chel 138; 10 p.p.m. | 8 (0·64) | 20 (0·89) | 25 (1·07) | 23 (1·17) | — |
| (*c*) | None | 9 (1·1) | — | — | — | 9 (1·08) |
| | EDTA; 10 p.p.m. | — | — | 14 (0·98) | 21 (1·11) | 23 (0·89) |
| (*d*) | None | 5 (—) | — | — | 9 (0·32) | — |
| | $FeSO_4$; 100 p.p.m. | — | 11 (—) | 16 (0·67) | 14 (0·40) | — |
| | $FeCl_3$; 10 p.p.m. | — | 5 (—) | 6 (0·39) | 6 (0·39) | — |

Table 8. *Changes in aconitase and chlorophyll content during recovery from iron deficiency*

Plants were grown in 24 crocks of nutrient solution containing 0·1 p.p.m. of iron as ferric EDTA and other nutrients as described in Table 4. From the two-leaf stage the plants in 12 crocks were given extra iron as ferric Chel 138 to a concentration of 2·5 p.p.m. Leaves 1 and 2 from one plant in each crock were pooled from each set of 12 crocks, and analysed for aconitase activity (μmoles of *cis*-aconitate formed/g. fresh wt./hr.) and chlorophyll content (μmoles/g. fresh wt.).

| | Expt. (*a*) | | | | | Expt. (*b*) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Aconitase | | Chlorophyll | | | Aconitase | | Chlorophyll | |
| Day of treatment | 0·1 p.p.m. of iron | 2·5 p.p.m. of iron | 0·1 p.p.m. of iron | 2·5 p.p.m. of iron | Day of treatment | 0·1 p.p.m. of iron | 2·5 p.p.m. of iron | 0·1 p.p.m. of iron | 2·5 p.p.m. of iron |
| 0 | 8 | 13 | 0·61 | 0·58 | 0 | 14 | 11 | 1·59 | 1·61 |
| 2 | 9 | 17 | 0·58 | 0·77 | 3 | 11 | 24 | 1·46 | 1·78 |
| 5 | 10 | 26 | 0·54 | 0·94 | 6 | 10 | 24 | 1·44 | 1·91 |
| 7 | 8 | 28 | 0·60 | 1·19 | 10 | 18 | 47 | 1·54 | 2·23 |
| 9 | 12 | 26 | 0·55 | 1·27 | 13 | 14 | 45 | 1·13 | 2·20 |
| 16 | 16 | 22 | 0·72 | 1·41 | | | | | |

plants a rise in aconitase activity in the treated leaves was usually apparent after 24 hr. (Table 7). The leaves used, although chlorotic, were by no means devoid of chlorophyll. The increase in aconitase did not seem to be dependent upon an increase in the chlorophyll content [cf. Expt. (*c*), Table 7].

*Feeding of iron to deficient plants.* If the concentration of iron in the nutrient solution was increased from 0·1 to 2·5 p.p.m. the chlorotic leaves gradually turned green, but, as with detached leaves, this increase in aconitase activity did not require a proportionate increase in the chlorophyll content and the two processes, although simultaneous, had no obvious connexion (Table 8). It will be noticed that the response of aconitase was seen in experiments (*a*) and (*b*) at quite different chlorophyll levels.

*Attempts to activate aconitase in homogenates*

If extracts were preincubated at 0° with 0·5 mM-ferrous ammonium sulphate and 0·01 M-cysteine for 1 hr. there was no increase in the aconitase activity. This was true of all the extracts tested, whether from iron-deficient or normal plants. In such experiments the concentrations of iron and cysteine in the subsequent assay were respectively 8 $\mu$M and 0·2 mM. When higher concentrations of iron (50 $\mu$M) and cysteine (1 mM) were present in the assay with *iso*citrate there was a stimulation of activity but this proved to be due to a non-enzymic reaction. When the aconitase activity was measured by the decrease in extinction with *cis*-aconitate as substrate the addition of iron and cysteine diminished the values obtained.

A similar effect was found with ascorbate. If ascorbate at concentrations equivalent to 500–1000 mg./100 g. of original leaf tissue was added to the extraction medium the aconitase activity measured with *iso*citrate appeared to be enhanced, but with *cis*-aconitate it was diminished. The presence of ascorbate equivalent to 100 mg./100 g. of leaf sample taken added about 0·06 to the change in extinction measured in 30 min. at 25°. This is equivalent to an aconitase activity of 12 $\mu$moles/g. fresh wt./hr. If the endogenous ascorbic acid of the leaf (50–100 mg./100 g.) is assumed to contribute in this way to the assay it would introduce an error of about 0·5–2·0 $\mu$moles/g. fresh wt./hr. under standard conditions.

Tests were also made of the results of mixing extracts prepared from plants of 0·2 and 5 p.p.m. iron concentrations. Extracts were mixed in various proportions, at least 30 min. at 0° being allowed before any mixture was tested. In five such experiments the following activities of mixtures were measured (in each case the value calculated from the activities of the separate extracts is given in parentheses) 13(11), 14(15), 22(29), 23(27), 37(40). It will be noted that, if anything, the activity of the mixture was less than that calculated.

## DISCUSSION

The results reported here show that when the iron supply to mustard plants in solution culture is so low that growth and chloroplast formation are affected, the aconitase activity extractable from each gram of leaf is markedly and consistently diminished. If the iron supply to the leaf is increased, the aconitase activity rises to the normal level.

That aconitase is specifically affected by iron deficiency is shown by the effects upon two other enzymes of the citric acid cycle: malic dehydrogenase is only slightly affected, and fumarase is, if anything, affected in the opposite way.

The variable results obtained at the highest concentration of iron used cannot yet be explained. The low average values obtained in certain experiments were partly due to the inclusion of extracts with no measurable aconitase activity. In seeking an explanation for these zero values, found especially in the experiments of Tables 2 and 3, it must be remembered that each extract tested was representative of leaves taken from nine to ten plants. On the other hand it may be significant that zero values were never obtained with plants of the 1 p.p.m. concentration, and only once (experiment of Table 6) with plants of the lowest concentration, despite the generally lower trend of these values. It is possible that as the leaves age they accumulate some inhibitory substance, but if this is so the inhibitor must have a specific action, because the malic-dehydrogenase activities (Table 3) were not affected.

With the evidence so far available it would seem that the lower aconitase activity in iron-deficient plants is due to a lack of the enzyme system as a whole and not merely due to a lack of iron. The activity of extracts from iron-deficient leaves was not restored by treatment with ferrous iron and cysteine, which has been shown repeatedly to activate aconitase from animal tissues (cf. Morrison 1954). It is conceivable that a particular complex of iron is required as an activator, but, if so, this substance is evidently not present in excess in normal leaves, because mixtures of extracts from normal and deficient plants did not show an activity greater than the sum of the activities measured separately. (Therefore there is still no direct evidence that iron is required for the action of aconitase in plants.)

The present experiments were undertaken to

test a hypothesis put forward by DeKock (1954–55) that in iron-deficient plants there is a deficiency of ferrous iron and that consequently the aconitase activity is diminished. A possible connexion between the effects upon aconitase and upon the concentrations of citric acid and malic acid and hence upon the phosphorus/iron and potassium/calcium ratios is further discussed by DeKock *et al.* (1960*b*). According to these arguments the concentration of citric acid should be raised and that of malic acid depressed, or at least the ratio of citric acid to malic acid should be greater in the leaves of iron-deficient plants. Experiments in progress (see also DeKock & Morrison, 1958) show this to be the case, but proof is still lacking that the lower level of aconitase is directly responsible. As we have already remarked (Bacon *et al.* 1961), it is difficult to decide, simply from a knowledge of its level of activity in extracts, whether an enzyme is a limiting factor in a sequence of metabolic reactions. What is required is a direct examination of the interconversion of organic acids in normal and deficient leaves.

## SUMMARY

1. Aconitase activity has been measured in extracts of the leaves of mustard plants growing in water culture and receiving three concentrations of iron (0·2, 1 and 5 p.p.m.).

2. The aconitase activity in plants receiving 0·2 p.p.m., which always show signs of iron-deficiency, was about half that in those receiving 1 p.p.m. At 5 p.p.m. the activity was variable, but usually greater than at 1 p.p.m.

3. Simultaneous measurements of malic-dehydrogenase activity showed that it was only slightly depressed by iron-deficiency; fumarase activity was slightly raised.

4. When iron-deficient plants or leaves were fed with adequate iron the aconitase activity was restored to the normal level.

5. The aconitase activity of extracts from deficient plants could not be increased by treatment with ferrous iron and cysteine, nor by any other means tested.

6. The significance of these results is discussed in relation to the possible connexion of iron with aconitase action, and to possible effects of aconitase deficiency on the metabolism of the leaf.

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# Some Enzymic Reactions Concerned in the Metabolism of Acetoacetyl-Coenzyme A in Athiorhodaceae

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Photosynthetic bacteria of the Athiorhodaceae family grow either aerobically in the dark or anaerobically in the light. Carotenoids and bacteriochlorophyll are formed only during growth in the light or under low conditions of aeration in the dark (van Niel, 1944; Cohen-Bazire, Sistrom & Stanier, 1957; Lascelles, 1959).

Organisms grown anaerobically in the light (pigmented) are considerably more active in catalysing the enzymic reactions concerned in the early stages of bacteriochlorophyll synthesis than are those cultivated aerobically in the dark (non-pigmented). The increase in activity, which is due to enhanced ability to synthesize the enzymes,

accompanies pigment formation in organisms adapting from the non-pigmented (aerobic) to the pigmented (photosynthetic) state (Lascelles, 1959, 1960). Similar changes in the activity of enzymes concerned in the early stages of carotenoid synthesis might also occur in Athiorhodaceae adapting from aerobic to photosynthetic conditions, and the present work was originally undertaken with this possibility in view.

There is considerable evidence that mevalonic acid is a precursor not only of cholesterol (Tavormina, Gibbs & Huff, 1957) but also of carotenoids (Braithwaite & Goodwin, 1957; Anderson, Nargard & Mangiarotti, 1959; Yokoyama, Chichester & Mackinney, 1960).

The early stages of carotenoid synthesis up to the formation of mevalonic acid probably proceed by the following pathway (in which CoA is coenzyme A, HMG β-hydroxy-β-methylglutaryl-, TPN triphosphopyridine nucleotide, and TPNH reduced triphosphopyridine nucleotide) (Ferguson, Durr & Rudney, 1959; Knappe, Ringlemann & Lynen, 1959):

$$2\ \text{Acetyl-CoA} \xrightleftharpoons{\beta\text{-ketothiolase}} \text{acetoacetyl-CoA} + \text{CoA} \quad (1)$$

$$\text{Acetoacetyl-CoA} + \text{acetyl-CoA} \xrightarrow[\text{enzyme}]{\text{HMG-CoA condensing}} \text{HMG-CoA} + \text{CoA} \quad (2)$$

$$\text{HMG-CoA} + \text{TPNH} \xrightarrow[\text{reductase}]{\text{HMG-CoA}} \text{mevalonic acid} + \text{TPN} \quad (3)$$

In addition to the enzymes concerned in this synthesis other reactions have been demonstrated in preparations from animal tissues and yeast which could compete for key intermediates. These include β-hydroxybutyryl-coenzyme A dehydrogenase (reaction 4) (Wakil, 1955); coenzyme A transferase (reaction 5) described by Stern, Coon, del Campillo & Schneider (1956*b*) and β-hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme (reaction 6) (Bachhawat, Robinson & Coon, 1955).

$$\beta\text{-Hydroxybutyryl-CoA} + \text{diphosphopyridine nucleotide} \rightleftharpoons \text{acetoacetyl-CoA} + \text{reduced diphosphopyridine nucleotide} \quad (4)$$

$$\text{Acetoacetyl-CoA} + \text{succinate} \rightleftharpoons \text{succinyl-CoA} + \text{acetoacetate} \quad (5)$$

$$\text{HMG-CoA} \longrightarrow \text{acetoacetate} + \text{acetyl-CoA} \quad (6)$$

Recent work has shown that acetoacetate formation by liver is due, at least in part, to cleavage of β-hydroxy-β-methylglutaryl-coenzyme A (Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958).

In the present work formation of free acetoacetate from acetyl-coenzyme A has been observed in extracts of Athiorhodaceae, suggesting the presence of some of the enzymes mentioned above. Their level of activity has been examined in extracts of pigmented and unpigmented organisms to see if there is a correlation between enzymic activity and ability to form carotenoids.

## EXPERIMENTAL

### *Materials*

CoA (70–75% pure), diphosphopyridine nucleotide (DPN) and TPNH were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; glutathione was obtained from British Drug Houses, Ltd., Poole, Dorset; β-hydroxy-β-methylglutaric acid and acetyl phosphate (dilithium salt) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Diketene was a gift from Dr D. Bigley, Dyson Perrins Laboratory, Oxford. Other chemicals were of the purest commercial grade available.

A partially purified preparation of phosphotransacetylase was made from an ultrasonic extract of *Escherichia coli* by a method similar to that of Goldman (1958). The preparation used in this work had an activity of 940 units/ml. (containing 47 mg. of protein) measured by arsenolysis of acetyl phosphate (Stadtman, 1955).

Acetyl-CoA was prepared by reaction of CoA and acetic anhydride (Simon & Shemin, 1953).

Acetoacetyl-CoA was prepared from diketene and CoA by the method described by Lynen *et al.* (1958).

β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA) was prepared from β-hydroxy-β-methylglutaric anhydride and CoA (Hilz, Knappe, Ringelmann & Lynen, 1958). The anhydride was made from the acid by the method of Hilz *et al.* (1958); its authenticity was established by melting-point determination and by chromatography of the hydroxamate derivative (Hilz *et al.* 1958). Acetoacetate was prepared from freshly distilled ethyl acetoacetate by hydrolysis of the ester with 2N-NaOH at 37° for 1 hr. (Krebs & Eggleston, 1945).

Tris buffers were prepared from 2-amino-2-hydroxymethylpropane-1:3-diol and HCl according to Gomori (1955).

### *Growth and harvesting of organisms*

*Rhodopseudomonas spheroides* N.C.I.B. no. 8253, *R. capsulata* van Niel, 2.3.11, and *R. palustris* van Niel, 2.1.7 were used. Stock cultures were maintained as described by Lascelles (1959).

*R. spheroides* was grown on malate plus glutamate medium (Lascelles, 1959), and *R. palustris* on the same medium supplemented with μM-*p*-aminobenzoate. For growth of *R. capsulata* sodium succinate (0·01 M) was used in place of malate.

Cultures were inoculated, incubated aerobically in the dark or anaerobically in the light, and harvested as described by Lascelles (1959). The washed organisms were finally suspended in 0·02M-tris buffer, pH 8·5, to a concentration of about 20 mg. dry wt./ml.

### *Preparation of cell-free extracts*

Extract of organisms were prepared by ultrasonic vibration (Lascelles, 1959); remaining whole organisms and debris were removed by centrifuging at 25 000*g* for 10 min.

at 0°. The supernatant fluid was used in all the work reported. Treatment of bacterial extract by Dowex 1 was effected by passing a portion of extract (equivalent to 15 mg. of protein) down a column (7·5 cm. × 1·5 cm.) of Dowex 1X8 (100–200 mesh; chloride form), the column having been cooled to 0°.

### *Estimations*

*Dry weight of organisms.* The extinctions of suspensions of the bacteria were measured at 680 mμ, and the dry weight was calculated from the calibration curve for the appropriate organism grown under the same conditions (Cohen-Bazire *et al.* 1957).

*Protein.* The concentration of protein in extracts was determined colorimetrically by a method based on the biuret reaction (Layne, 1957) after removal of photosynthetic pigments by treatment with hot acid–ethanol (Vernon & Kamen, 1953). Crystalline bovine plasma albumin (Armour Laboratories, Hampden Park, Eastbourne, Sussex) was the standard.

*Photosynthetic pigments.* Carotenoids and bacteriochlorophyll were estimated by the method of Cohen-Bazire *et al.* (1957).

*Acetoacetate.* This was estimated colorimetrically as the *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan, formed after reaction with an excess of *p*-nitrobenzenediazonium hydroxide (Walker, 1954).

### *Spectrophotometry*

All spectrophotometric measurements were with the Unicam spectrophotometer model SP. 500 (Unicam Instruments Ltd., Cambridge).

### *Assay of enzymic activities*

*β-Ketothiolase.* The assay of this enzyme was based upon measurement of the decline in absorption at 310 mμ due to acetoacetyl-CoA in the presence of $Mg^{2+}$ ions, which enhances the absorption of the enolate ion of the β-keto thio ester (Stern, 1955, 1956). The assay was conducted in silica microcuvettes (light-path of 1 cm.) containing (in μmoles): tris buffer, pH 8·5, 75; $MgCl_2$, 5; CoA, 0·01; glutathione, 3; acetoacetyl-CoA, 0·02; bacterial extract, 1–5μg. of protein; water to a final volume of 0·4 ml. The reaction was started by the addition of the enzyme and the absorption at 310 mμ measured at half-minute intervals against a control which lacked acetoacetyl-CoA. The reaction was carried out at room temperature (about 22°).

The enzyme activity was expressed in arbitrary units, one unit causing an initial rate of decline in $E_{310\ m\mu}$ of 0·01/min.

*Coenzyme A transferase (succinyl-β-oxoacyl-S-coenzyme A transferase.)* The procedure for this assay is based on that for the enzyme in pig heart (Stern *et al.* 1956*b*), except that the breakdown, rather than formation, of acetoacetyl-CoA was determined by measuring the decline in $E_{310\ m\mu}$. The assay was carried out in silica microcuvettes (light-path 1 cm.) containing (in μmoles): tris buffer, pH 8·5, 75; $MgCl_2$, 5; acetoacetyl-CoA, 0·02; sodium succinate, 5; bacterial extract, 5–30μg. of protein; water to a final volume of 0·4 ml. The reaction was started by the addition of succinate and the decline in absorption measured against a control lacking acetoacetyl-CoA. The assay was conducted at room temperature (about 22°).

One unit of enzyme activity was the amount causing a rate of decline in $E_{310\ m\mu}$ of 0·01/min.

*β-Hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme.* The cleaving of HMG-CoA to form equimolar amounts of acetoacetate and acetyl-CoA was assayed by estimation of the acetoacetate formed. Tubes (1 cm. diameter) contained (in μmoles): tris buffer, pH 8·5, 200; HMG-CoA, 0·20; bacterial extract, 1 mg. of protein; water to a final volume of 0·9 ml. The reaction was started by the addition of the bacterial extract, and after incubation for 30 min. at 34° 0·1 ml. of 30% trichloroacetic acid was added. After removal of the precipitate by centrifuging, acetoacetate was estimated by the method of Walker (1954).

This method, which depends upon the reaction of acetoacetic acid with excess of *p*-nitrobenzenediazonium hydroxide, is, under the conditions employed, essentially specific for this compound (Walker, 1954; Drummond & Stern, 1960). Acetoacetyl-CoA does not form the coloured compound, *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan, produced by acetoacetate (Lynen *et al.* 1958; Drummond & Stern, 1960).

Activity is expressed as μmoles of acetoacetate formed/hr./mg. of protein, under the standard conditions.

*β-Hydroxybutyrate dehydrogenase.* The assay of D(−)-β-hydroxybutyrate dehydrogenase was based upon measurement of the amount of reduced diphosphopyridine nucleotide (DPNH) formed from DPN in the presence of β-hydroxybutyrate.

Silica microcuvettes (light-path 1 cm.) contained (in μmoles): tris buffer, pH 8·5, 75; DPN, 0·35; sodium β-hydroxybutyrate, 1·0; bacterial extract, 50–200μg. of protein; water to a final volume of 0·4 ml. The addition of the bacterial extract initiated the reaction and the increase in $E_{340\ m\mu}$ was recorded. The formation of DPNH, taking readings at 30 sec. intervals, was linear over the range 50–250μg. of protein for the first 4 min.

One unit of activity was the amount causing an increase of 0·01/min. in $E_{340\ m\mu}$.

## RESULTS

### *Synthesis of photosynthetic pigments by Athiorhodaceae under different growth conditions*

The concentration of bacteriochlorophyll in *R. spheroides* and *R. palustris* grown aerobically in the dark under the conditions used in this work was about 1% of that in photosynthetically grown organisms (Table 1). Aerobically grown *R. capsulata* was more pigmented than the other organisms when grown under comparable conditions, containing about 4% of the amount of bacteriochlorophyll found in photosynthetically grown organisms. The levels of carotenoid were also considerably reduced by growth under aerobic conditions (Table 1).

### *Formation of acetoacetate*

Extracts of *R. spheroides*, *R. capsulata* and *R. palustris* formed acetoacetate when incubated with acetyl-CoA (generated from acetyl phosphate by phosphotransacetylase) and succinate (Table 2).

Table 1. *Bacteriochlorophyll and carotenoid content of Athiorhodaceae grown anaerobically in the light and aerobically in the dark*

| Organism | Growth conditions | Bacteriochlorophyll | Carotenoid |
|---|---|---|---|
| | | (μm-moles/mg. dry wt.) | |
| *R. spheroides* | Anaerobic, light | 23·0 | 6·0 |
| | Aerobic, dark | 0·3 | 0·2 |
| *R. capsulata* | Anaerobic, light | 34·0 | 9·0 |
| | Aerobic, dark | 1·4 | 1·4 |
| *R. palustris* | Anaerobic, light | 24·0 | —* |
| | Aerobic, dark | 0·2 | — |

* Not examined.

Table 2. *Acetoacetate formation by extracts of Athiorhodaceae*

Each tube (1 cm. diam.) contained: bacterial extract, 1 mg. of protein; tris buffer, pH 8·5, 100 μmoles; acetyl phosphate, 25 μmoles; sodium succinate, 10 μmoles; CoA, 0·05 μmole; $Na_2S$, 4 μmoles; phosphotransacetylase, 5 units; water to 0·9 ml. Tubes were incubated at 34° for 30 min. and the reaction was stopped by addition of 0·1 ml. of 30% trichloroacetic acid. The precipitate was removed by centrifuging and the acetoacetate determined as described in the Experimental section. The activity is expressed as μmole of acetoacetate formed/hr./mg. of protein. The results are the average of three determinations with each type of extract; the range is shown in parentheses.

| Organism | Acetoacetate synthesis by extracts from organisms grown: Aerobic, dark | Anaerobic, light |
|---|---|---|
| *R. capsulata* | 0·32 (0·20–0·40) | 0·15 (0·14–0·16) |
| *R. spheroides* | 0·20 (0·16–0·26) | 0·10 (0·08–0·12) |
| *R. palustris* | 0·23 (0·18–0·28) | 0·09 (0·09) |

The amount formed/mg. of protein under the standard conditions was two to three times higher with extracts from aerobically grown bacteria than with those from organisms grown anaerobically in the light (Table 2).

Synthesis of acetoacetate was examined in more detail with extracts of aerobically grown *R. capsulata*.

*Requirements for synthesis.* When the extracts were incubated in the complete reaction mixture (containing tris buffer, acetyl phosphate, succinate, CoA, sodium sulphide and phosphotransacetylase) acetoacetate was formed at a linear rate over a period of 30 min. provided that the final protein concentration was from 0·5 to 3 mg./ml. (Fig. 1). The optimum pH for synthesis was about 8·5.

No acetoacetate was formed when acetyl phosphate was omitted from the reaction mixture and synthesis was reduced to about 20% in the absence of succinate (Table 3). Little synthesis

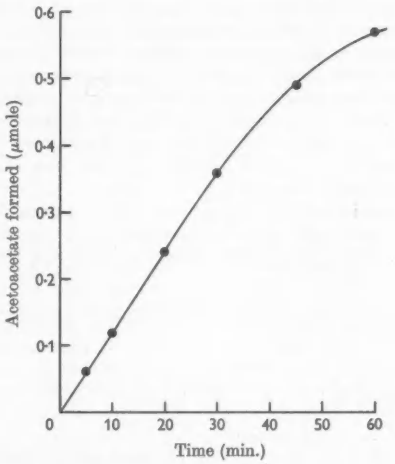


Fig. 1. Production of acetoacetate by *Rhodopseudomonas capsulata* extract (1·6 mg. of protein). Incubation conditions as in Table 2.

Table 3. *Requirement for acetoacetate formation by extracts of* Rhodopseudomonas capsulata

The complete system contained: extract of aerobically grown *R. capsulata*, 0·1 ml. (1·5 mg. of protein); tris buffer, pH 8·5, 100 μmoles; acetyl phosphate, 25 μmoles; sodium succinate, 10 μmoles; CoA, 0·05 μmole; $Na_2S$, 4 μmoles; phosphotransacetylase, 5 units; water to 0·9 ml. Incubation was for 30 min. at 34° before estimation of the acetoacetate as described (Table 2).

| Component omitted | Acetoacetate formed (μmole) |
|---|---|
| None | 0·44 |
| Acetyl phosphate | 0 |
| Succinate | 0·07 |
| CoA | 0·03 |
| Phosphotransacetylase | 0·01 |

occurred in the absence of CoA, and added phosphotransacetylase was also necessary (Table 3); the activity of this enzyme is only slight in these extracts. In the crude extracts stimulation by sulphide or glutathione was observed only occasionally. Glutathione, however, was needed for maximum synthesis by extracts treated with Dowex 1. It is also required for acetoacetate formation by liver preparations (Lynen *et al.* 1958).

Addition of adenosine di- or tri-phosphate or of inorganic phosphate (each at a concentration of 5 $\mu$moles/ml.) did not increase acetoacetate formation.

*Identification of acetoacetate.* The method used to estimate acetoacetate involves its coupling with *p*-nitrobenzenediazonium hydroxide to give *NN'*-bis(nitrophenyl)-*C*-acetylformazan, which has an absorption maximum at 450 m$\mu$ (Walker, 1954). After reaction with *p*-nitrobenzenediazonium hydroxide the compound formed in the enzymic reaction mixture had the same absorption spectrum (over the range 300–600 m$\mu$) as that given by authentic acetoacetate treated under the same conditions.

Identity of the product with acetoacetate was confirmed also by chromatography of the 2:4-dinitrophenylhydrazones (Dagley, Fewster & Happold, 1952).

*Enzymes concerned in the formation of acetoacetate*

The stimulation by succinate of acetoacetate formation from acetyl-CoA suggested that the CoA-transferase enzyme might participate; this would catalyse acetoacetate formation from acetoacetyl-CoA, arising from acetyl-CoA by the action of $\beta$-ketothiolase. The activity of these enzymes were therefore examined in extracts of Athiorhodaceae.

*$\beta$-Ketothiolase.* This enzyme was highly active in extracts of all the organisms tested and no difference was observed in preparations from cultures grown in the light or dark (Table 4). Even with crude extracts omission of CoA reduced the activity to about 15% of that in the complete system, whereas treatment with Dowex 1 reduced the activity in the absence of added CoA to about 5%. The inability to observe complete dependence upon CoA could have been due to traces of this substance in the acetoacetyl-CoA used as substrate.

*Coenzyme A transferase.* This enzyme was present in extracts of all three organisms tested. Its activity in extracts from both *R. spheroides* and *R. capsulata* grown anaerobically in the light was about 40% less than that found in preparations from aerobic cultures (Table 4). This difference in activity, though slight, was observed consistently.

The enzyme was examined in more detail with extracts of aerobically grown *R. spheroides*. The optimum pH was from 8·0 to 8·5, and succinate was not replaced by malate or fumarate. High concentrations of succinate were needed for maximum activity; this is in accord with observations made with purified preparations from pig heart (Stern *et al.* 1956*b*). In the present experiments the succinate concentration was 5 $\mu$moles/0·4 ml., which permitted the enzymic reaction to proceed at nearly optimum rate. Higher concentrations of succinate interfered with the assay by chelating with $Mg^{2+}$ ions (Stern *et al.* 1956*b*), thereby reducing their availability for complex formation with acetoacetyl-CoA, on which the sensitivity of the assay depends.

*Enzymes concerned in the metabolism of $\beta$-hydroxy-$\beta$-methylglutaryl-coenzyme A*

Key enzymes in the cycle proposed by Lynen *et al.* (1958) to account for the formation of free acetoacetate in liver are the HMG-CoA condensing enzyme (reaction 2) and the HMG-CoA cleavage enzyme (reaction 6). Evidence for the presence of these was sought in extracts of *R. spheroides* and *R. capsulata*.

Table 4. *$\beta$-Ketothiolase and succinyl-coenzyme A transferase in extracts of Athiorhodaceae*

Extracts were assayed under the standard conditions described in the Experimental section. The activities are expressed as units of enzyme/10 $\mu$g. of protein. The results are the mean values of several estimations with different extracts; the figures in parentheses show the range and the number of determinations.

| Organism | Growth conditions | Activity: $\beta$-Ketothiolase | Activity: CoA transferase |
|---|---|---|---|
| *R. spheroides* | Anaerobic, light | 40 (34–50; 3) | 3·3 (2·6–4·0; 4) |
| | Aerobic, dark | 40 (30–50; 3) | 5·3 (4·0–5·7; 4) |
| *R. capsulata* | Anaerobic, light | 35 (30–40; 2) | 2·7 (2·3–3·4; 3) |
| | Aerobic, dark | 30 (25–35; 2) | 5·0 (4·0–6·0; 5) |
| *R. palustris* | Anaerobic, light | —* | 2·7 (1 determination only) |
| | Aerobic, dark | — | 4·1 (1 determination only) |

* Not tested.

*β-Hydroxy-β-methylglutaryl-coenzyme A condensing enzyme.* This enzyme has been assayed in purified preparations from yeast (Ferguson & Rudney, 1959) and animal tissues (Lynen *et al.* 1958) by observing the rate of decline in $E_{310\ m\mu}$ due to condensation of acetoacetyl-CoA with acetyl-CoA. This method was applied to crude extracts of *R. capsulata* grown aerobically in the dark. However, the crude extracts, being rich in β-ketothiolase, rapidly decomposed acetoacetyl-CoA and the rate of decline in $E_{310\ m\mu}$ was not increased by addition of acetyl-CoA.

Treatment of extracts with Dowex 1 reduced the β-ketothiolase activity to about 5 % in the absence of added CoA, but such extracts also failed to give evidence of the presence of HMG-CoA condensing enzyme.

β-Ketothiolase is more sensitive to inhibition by iodoacetamide than the HMG-CoA condensing enzyme and this differential effect has been used to eliminate β-ketothiolase activity from partially purified preparations of the condensing enzyme (Lynen *et al.* 1958; Ferguson & Rudney, 1959). Treatment of the bacterial extracts at 0° with iodoacetamide as described by Ferguson & Rudney (1959), or addition of the inhibitor (2–10 mM) directly to the assay mixture, was unsuccessful. The preparations were either completely inactivated or else there was no stimulation of acetoacetyl-CoA disappearance by acetyl-CoA.

Attempts to destroy differentially the β-ketothiolase by heat treatment also failed.

The presence of HMG-CoA could not be detected chromatographically (as the hydroxamate) in the incubation mixtures used to demonstrate free acetoacetate formation. In these experiments hydroxylamine (0·5 M, final concentration) was added after incubation for 60 min., and the hydroxamates were isolated and chromatographed (Stadtman & Barker, 1950); only acetohydroxamate was detected.

*β-Hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme.* Extracts of *R. spheroides*, *R. capsulata* and *R. palustris* formed acetoacetate when incubated with HMG-CoA, suggesting the presence of the HMG-CoA cleavage enzyme (Bachhawat *et al.* 1955). Extracts from aerobically grown *R. spheroides* were about three times more active than those from photosynthetically grown bacteria (Table 5).

The enzyme system was examined in more detail in extracts of aerobically grown *R. capsulata*. Addition of $Mg^{2+}$ ions or glutathione did not increase its activity though both are required by the purified preparations from pig heart (Bachhawat *et al.* 1955). The optimum pH was at least 9·1, the highest pH tested. The cleavage enzyme in pig heart is also favoured by alkaline conditions, maximum activity occurring at pH 10 (Bachhawat *et al.* 1955).

To confirm that the acetoacetate formed from HMG-CoA was due to the cleavage enzyme, evidence for the other product of the reaction, acetyl-CoA, was sought with extracts of aerobically grown *R. capsulata*. To demonstrate the presence of acetyl-CoA use was made of the malate synthetase which is present in *R. capsulata* (Kornberg & Lascelles, 1960). When assayed by the method of Dixon & Kornberg (1959) the extracts were shown to be capable of forming malate from acetyl-CoA and glyoxylate at the rate of 2·8 μmoles/hr./mg. of protein. Incubation of extracts with [1-$^{14}$C]glyoxylate and subsequent radioautography showed incorporation of isotope into two components and that this was dependent on the presence of HMG-CoA (Table 6). Elution of these areas from the chromatogram and subsequent co-chromatography established their identity with malate and citrate. The latter evidently arose from the further metabolism of malate.

Table 5. *Cleavage of β-hydroxy-β-methylglutaryl-coenzyme A by extracts of Athiorhodaceae*

Bacterial extracts were incubated with HMG-CoA under the conditions described in the Experimental section. Activity is expressed as μmole of acetoacetate formed/hr./mg. of protein, and each value is the mean of three estimations with each type of extract. The figures in parentheses show the range.

| Organism | Growth conditions | Activity |
|---|---|---|
| *R. spheroides* | Aerobic, dark | 0·15 (0·09–0·21) |
| | Anaerobic, light | 0·04 (0·03–0·05) |
| *R. capsulata* | Aerobic, dark | 0·10 (0·07–0·11) |

Table 6. *Synthesis of* [$^{14}$C]*malate by extracts of* Rhodopseudomonas capsulata

The complete reaction mixture contained: extract of aerobically grown *R. capsulata*, 4·5 mg. of protein; tris buffer, pH 8·5, 400 μmoles; $MgCl_2$, 5 μmoles; sodium [1-$^{14}$C]glyoxylate, 0·2 μmole (0·5 μc, 50 000 counts/min.); HMG-CoA, 0·5 μmole; water to 1·0 ml. Incubation was for 1 hr. at 34° in 1 cm. tubes and the reaction terminated by addition of 2 ml. of ethanol. In the control, ethanol was added at zero time. The ethanol extracts were examined by radioautography as described by Kornberg (1958).

| Experimental conditions | Counts incorporated into malate and citrate* (%) |
|---|---|
| Complete reaction mixture | 16·4 |
| Without HMG-CoA | 0·80 |
| Zero-time control | 0·20 |

* Percentage of total counts applied to chromatogram appearing in the malate and citrate.

*β-Hydroxybutyrate dehydrogenase*

Extracts of *R. spheroides*, *R. capsulatus* and *R. palustris* reduced DPN in the presence of DL-β-hydroxybutyrate. Only the D-isomer was active in this respect (personal communication from Professor H. A. Krebs). The activity of all organisms was similar and there was no significant difference in extracts prepared from cultures grown under the different conditions (Table 7). This enzyme was freely reversible, oxidizing DPNH in the presence of free acetoacetate.

*β-Hydroxybutyryl-coenzyme A dehydrogenase*. The presence of this enzyme was demonstrated in extracts of *R. spheroides* by the decline in $E_{340\ m\mu}$, due to DPNH in the presence of acetoacetyl-CoA. The assay of the enzyme was complicated by DPNH oxidase and β-ketothiolase present in the bacterial extract and no accurate measure of activity could be obtained from crude preparations.

## DISCUSSION

The activities of the enzymes examined were similar in extracts of pigmented and non-pigmented organisms. There was no evidence that their levels contributed to the regulation of carotenoid synthesis by these bacteria. It has been suggested that formation of HMG-CoA is a key reaction in the synthesis of cholesterol and that at this point of formation of the basic branched carbon unit some control of cholesterol synthesis may be effected (Rudney & Ferguson, 1959; Lynen *et al.* 1958). This might also apply to carotenoid formation by the photosynthetic bacteria. The HMG-CoA condensing enzyme could not be demonstrated in crude extracts of these organisms, even after treatment with iodoacetamide, but its presence certainly cannot be excluded. This enzyme in liver (Lynen *et al.* 1958) can only be assayed spectrophotometrically in partially purified preparations. The presence in the bacterial extracts of a highly active β-ketothiolase, which would compete for the common substrate, acetoacetyl-CoA, accentuates the difficulty of demonstrating the HMG-CoA condensing enzyme. In addition, the HMG-CoA cleavage enzyme present in the extracts could also contribute to the inability to detect the condensing enzyme by the methods used. Recently evidence has been provided that the activity of the HMG-CoA reductase (reaction 3) is a major factor in the control of cholesterol formation by liver (Bucher, Overath & Lynen, 1960). Attempts to demonstrate this enzyme spectrophotometrically in extracts of *R. spheroides* by the decline in $E_{340\ m\mu}$ due to TPNH oxidation have been unsuccessful, but this negative result may not be significant since only crude extracts were used.

Table 7. *β-Hydroxybutyrate dehydrogenase in extracts of Athiorhodaceae*

Extracts were assayed as described in the Experimental section. The results are expressed as units/μg. of protein. The activity shown is for one extract of each type.

| Organism | Growth conditions | Activity |
|---|---|---|
| *R. spheroides* | Aerobic, dark | 0·096 |
| | Anaerobic, light | 0·140 |
| *R. capsulata* | Aerobic, dark | 0·079 |
| | Anaerobic, light | 0·052 |
| *R. palustris* | Aerobic, dark | 0·090 |
| | Anaerobic, light | 0·140 |

The formation of acetoacetate by extracts of Athiorhodaceae under the conditions used may be attributable to the action of β-ketothiolase (reaction 1) and CoA transferase (reaction 5). Stimulation of acetoacetate formation by succinate supports such a mechanism, which has been demonstrated in preparations from ox heart (Stern, Coon & del Campillo, 1956*a*). In liver there is evidence that acetoacetate also arises by cleavage of HMG-CoA (Lynen *et al.* 1958) and by deacylation of acetoacetyl-CoA (Drummond & Stern, 1960).

The level of β-ketothiolase in the photosynthetic bacteria is strikingly high, the activity (1·2 μmoles decomposed/mg./min.) being only slightly less than that in extracts of *Clostridium kluyveri* (2 μmoles/mg./min.), in which it is a key enzyme in the energy-yielding mechanism of the organism (Barker, 1956). The activity is three times as high as in *Mycobacterium tuberculosis* (0·4 μmole/mg./min.) (Goldman & Gelbard, 1959). The β-ketothiolase links the tricarboxylic acid cycle and fat metabolism. The high activity in the photosynthetic bacteria might be connected with the metabolism of the polymer of β-hydroxybutyric acid, an assimilation product which comprises up to 25 % of their dry weight under certain conditions (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959). It has been suggested that the β-hydroxybutyryl-CoA dehydrogenase participates in the formation of the polymer from acetoacetyl-CoA (Stanier *et al.* 1959) and this enzyme was found in the bacterial extracts. However, the D(−)-hydroxybutyrate dehydrogenase, examined in preparations from animal tissues (Wakeman & Dakin, 1909; Green, Dewan & Leloir, 1937; Lehninger, Sudduth & Wise, 1960) is highly active in extracts of Athiorhodaceae. It may possibly be connected with the metabolism of the polymer, thus affording an explanation of its relatively high activity.

β-Ketothiolase, L(+)-β-hydroxybutyryl-CoA dehydrogenase and a high level of crotonase have been shown in extracts of *Rhodospirillum rubrum*

(Stern, del Campillo & Raw, 1956); these enzymes might also reflect the ability of this organism to form polymer (Stanier *et al.* 1959).

## SUMMARY

1. Cell-free extracts of photosynthetic bacteria of the Athiorhodaceae family form acetoacetate from acetyl phosphate in the presence of phosphotransacetylase and coenzyme A. Such extracts are rich in β-ketothiolase and coenzyme A transferase.

2. The extracts contained β-hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme. Neither the formation of β-hydroxy-β-methylglutaryl-coenzyme A from acetoacetyl-coenzyme A and acetyl-coenzyme A, nor its reduction to mevalonic acid by reduced triphosphopyridine nucleotide, was detected.

3. The level of activity of these enzymes, and of β-hydroxybutyrate dehydrogenase and β-hydroxybutyryl-coenzyme A dehydrogenase, did not differ significantly in extracts prepared from pigmented and non-pigmented organisms.

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# The Estimation of 4-Hydroxy-3-Methoxymandelic Acid in Urine

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Armstrong & McMillan (1957) identified 4-hydroxy-3-methoxymandelic acid (vanilmandelic acid) in normal human urine and demonstrated that increased quantities were excreted after noradrenaline infusion and by patients with the catecholamine-secreting tumour, phaeochromocytoma. Subsequent work has established that 3-methoxylation followed by oxidative deamination to 4-hydroxy-3-methoxymandelic acid is a major metabolic pathway of both noradrenaline and adrenaline (see Axelrod, 1959). Some 4-hydroxy-3-methoxymandelic acid is also formed by an alternative pathway in which oxidative deamination precedes 3-methoxylation (Kopin, 1960).

About 45% of the total noradrenaline and adrenaline production in man is excreted as 4-hydroxy-3-methoxymandelic acid (Kopin, 1960) compared with a free-amine excretion of only 1–4% (Euler & Luft, 1951; Goldenberg, Serlin, Edwards & Rapport, 1954). Estimation of small concentrations of free catecholamines in urine demands specialized skills and equipment for the biological or fluorimetric procedures involved; results are often conflicting and depend on the method of assay (Goldenberg *et al.* 1954). Catechol compounds generally are inherently unstable. It is thus likely that 4-hydroxy-3-methoxymandelic acid, by virtue of its stability and relatively large output, will provide a better guide to noradrenaline and adrenaline turnover, if a simple technique for its assay were available. A number of quantitative techniques have been described (Armstrong, Shaw & Wall, 1956; Robinson, Ratcliffe & Smith, 1959; Smith, Schweitzer & Wortis, 1959; Weise, McDonald & LaBrosse, 1960; Studnitz & Hanson 1959) but none of these methods is well suited to the clinical laboratory.

We have given preliminary reports of a colorimetric method for 4-hydroxy-3-methoxymandelic acid assay (Sandler & Ruthven, 1959*a*, *b*). The modification described here is both simpler and more specific. It involves the absorption of urinary 4-hydroxy-3-methoxymandelic acid on an anion-exchange resin, and its elution, extraction and subsequent conversion into vanillin, which is measured spectrophotometrically. Apart from its value in the diagnosis of phaeochromocytoma, it is suitable for the study of small changes in 4-hydroxy-3-methoxymandelic acid output at normal excretion levels.

## EXPERIMENTAL

*Materials.* Acetate buffers were prepared from ammonium acetate and acetic acid. Vanillin standard was prepared daily by diluting 0·2 ml. of an aq. stock solution of vanillin (A.R.) (313 mg./l.) to 25 ml. with 0·23 N-HCl (A.R.). The stock solution was stored at 4° and was prepared once a month. Aq. solution of sodium borohydride (10%, w/v) was freshly prepared just before use. Ethyl acetate (for spectroscopic use, British Drug Houses Ltd.) and alumina (aluminium oxide M.F.C. purified neutral, Hopkin and Williams Ltd.) were used.

Dowex analytical-grade anion-exchange resin AG1-X2 (200–400 mesh; Cl form) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was converted into its acetate form. The resin (100 g.) was slurried with 4 M-ammonium acetate, pH 5·9 (50 ml.) and the fines were aspirated after standing for 10 min. The creamy mixture was poured into a column (2·5 cm. × 50 cm.), the excess of fluid drained off, and the packed resin eluted with 4 M-ammonium acetate, pH 5·9 (about 400 ml.) until the eluate was almost chloride-free. The excess of buffer was then removed by washing the column with water (about 500 ml.) and, after draining, the resin was extruded and stored damp at 4°.

*Procedure.* From 1·0 ml. (or 0·5 ml., see below) of urine diluted with 1 ml. of water an autoclaved sample was prepared as described by Sandler & Ruthven (1959*a*) except that Dowex AG1-X2 (acetate form) was used, the ethyl acetate extract was evaporated in a stream of $N_2$ instead of *in vacuo*, and the evaporated extract was dissolved in 0·23 N-HCl instead of 5% $H_2SO_4$. A tube containing 2·5 ml. of vanillin standard and 0·2 g. of alumina was autoclaved at the same time. A 2·0 ml. portion of the autoclaved solution was adjusted with 0·4 ml. of 2 M-diethanolamine to approx. pH 9·1. The precipitate of $Al(OH)_3$ which formed removed any chromogens present and was separated by centrifuging. The clear colourless supernatant liquid was transferred to a 10 mm. cuvette (capacity 3·5 ml.). The partly filled cell was placed in its carrier with its base raised 5 mm. to ensure an unobstructed light-path, and the extinction read against water at 365 and 380 mμ. Sodium borohydride solution (approx. 0·1 ml.) was then added and after 30 min. the extinction redetermined at 365 and 380 mμ in the same cell. Obstruction of the light-path by bubbles of $H_2$ from the borohydride was avoided by using cells freshly cleaned in chromic acid and by dislodging any bubbles that did form by sharply tapping the cuvette with the finger just before reading. The autoclaved vanillin standard was treated in a similar way. If the extinctions at wavelength $\lambda$ of autoclaved urine extracts before and after

treatment with sodium borohydride are indicated by $T_\lambda$ and $TB_\lambda$ and the corresponding extinctions of autoclaved vanillin standards by $S_\lambda$ and $SB_\lambda$, the amount of 4-hydroxy-3-methoxymandelic acid in the urine specimen (1 or 0·5 ml.) is

$$\frac{8{\cdot}15\,[(T_{365}-1{\cdot}05TB_{365})-(T_{380}-1{\cdot}05TB_{380})]}{(S_{365}-1{\cdot}05SB_{365})-(S_{380}-1{\cdot}05SB_{380})}\ \mu g.$$

*Collection of urine samples.* Specimens (24 hr.) were acidified with 25 ml. of acetic acid (A.R.). Although 4-hydroxy-3-methoxymandelic acid is very stable in urine, decomposition of bicarbonate during elution of an alkaline urine tends to cause disruption of the column.

### *Comments on the method*

The eluate from two columns, one containing 100$\mu$g. of 4-hydroxy-3-methoxymandelic acid in 1 ml. of water and the other 100$\mu$g. in 1 ml. of urine, was collected in 1 ml. fractions. The peak level of 4-hydroxy-3-methoxymandelic acid was found in the same fraction (9 ml.) in each column.

*Extraction of 4-hydroxy-3-methoxymandelic acid from acetate buffer.* Only about 5% of 4-hydroxy-3-methoxymandelic acid was extracted from acetate buffer with equal volumes of ethyl acetate, with or without saturation with NaCl; however, acidification (pH < 1) and saturation with NaCl caused about 75% of the 4-hydroxy-3-methoxymandelic acid to pass into the organic phase. Two extractions with ethyl acetate removed over 90%. Ether extraction was not as satisfactory (recovery approx. 65% after two extractions). Less than 3% loss of 4-hydroxy-3-methoxymandelic acid (10$\mu$g.), added to ethyl acetate (15 ml.) previously equilibrated with acetate buffer, was detected when the solvent was removed under vacuum between 40° and 100°. When ethyl acetate was removed by a stream of $N_2$, recoveries of 4-hydroxy-3-methoxymandelic acid from urine were of the same order (65–75%) as those obtained when the solvent was evaporated under vacuum.

*Paper-chromatographic identification of vanillin.* Autoclaved extracts of normal urine or of urine from patients with phaeochromocytoma, prepared without alumina, were evaporated to dryness at 37° in an incubator. The residues were chromatographed on Whatman no. 1 filter paper by the ascending method in propan-2-ol–aq. $NH_3$ soln. (sp.gr. 0·88)–water (20:1:2, by vol.). Two-dimensional chromatograms were also prepared with butan-1-ol–pyridine–water (10:3:3, by vol.) as second solvent. Papers were dried in a stream of warm air and dipped in a mixture of 0·003% (w/v) indole in ethanol–orthophosphoric acid (3:5, v/v) (Sandler & Ruthven, 1959*a*). Vanillin, which was invariably present in urine extracts from normal subjects and present in increased concentration in phaeochromocytoma urine extracts, appeared as a pink spot ($R_F$ 0·5 in the propan-2-ol system; 0·8 in the butan-1-ol system). *p*-Hydroxybenzaldehyde, usually present in trace amounts which were not increased in phaeochromocytoma, showed as an orange spot ($R_F$ 0·6 in the propan-2-ol system; 0·85 in the butanol system). These were the only substances detected except for a purple spot, shown to be an artifact, near the origin in both solvent systems and an occasional faint-brown spot that developed late ($R_F$ 0·3 in the propan-2-ol system; 0·2 in the butanol system).

*Spectrophotometric estimation of vanillin.* The absorption max. of vanillin shifts to a higher wavelength with an increase in pH (Englis & Wallerman, 1957). Vanillin in diethanolamine buffer, pH 9·1, has an absorption max. in water at 347 m$\mu$ (Fig. 1). *p*-Hydroxymandelic acid, present in normal urine (Armstrong *et al.* 1956), is converted during autoclaving into *p*-hydroxybenzaldehyde, which has an absorption max. at 330 m$\mu$ (Fig. 1). Dihydroxymandelic acid, which occurs in urine (Goodall, 1959) would presumably be similarly converted into dihydroxybenzaldehyde in the autoclave. This compound could not be detected, however, in autoclaved extracts of urine to which the parent acid had been added.

Advantage has been taken of the rapid and approximately linear change in extinction of vanillin between 360 and 380 m$\mu$ (Fig. 1) to increase the specificity of the method. Thus the three values obtained when the 4-hydroxy-3-methoxymandelic acid concentration of urine was calculated at 360–380 m$\mu$, 365–380 m$\mu$ and 370–380 m$\mu$ agreed closely with each other (15 experiments). Further precision has been obtained by measuring extinction before and after the addition of sodium borohydride to the alkaline solution. Vanillin is reduced and its reduction products show negligible absorption at 365 or 380 m$\mu$ (Pisano, 1960). Under the conditions used, vanillin obeys Beer's Law up to an extinction difference (365–380 m$\mu$) of 0·8.

When the observed curve of the autoclaved extract from a typical normal urine (curve *A*, Fig. 2*a*) was corrected for non-reducible material (curve *E*) the resultant curve (*B*) did not coincide in shape or height with a theoretical curve (*D*) for vanillin at a concentration calculated to be present in the extract. Curve *D* was constructed from the known curve of pure vanillin at a concentration equal to that obtained by applying the formula given above to the relevant extinction measurements of the extract. This

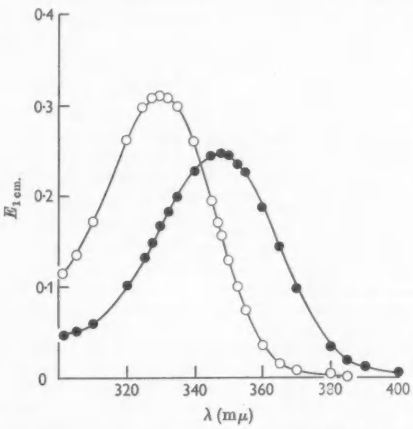


Fig. 1. Absorption spectra of vanillin (●), ($\lambda_{max.}$ 347 m$\mu$), and *p*-hydroxybenzaldehyde (○), ($\lambda_{max.}$ 330 m$\mu$). The compounds (1·4$\mu$g./ml.) were dissolved in 2 ml. of 0·23 N-HCl and the solutions adjusted to about pH 9·1 with 0·4 ml. of 2 M-diethanolamine.

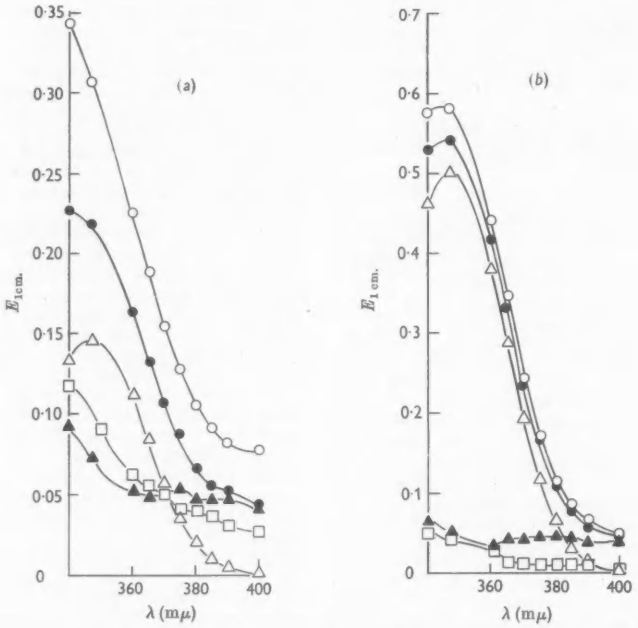


Fig. 2. Absorption spectra derived from autoclaved extracts (pH 9·1) from (*a*) normal urine and (*b*) phaeochromocytoma. Curve *A* (○), before reduction; curve *B* (●), borohydride reducible substances; curve *C* (▲), non-vanillin reducible substances; curve *D* (△), vanillin constructed by calculation from formula; curve *E* (□), non-reducible substances.

Table 1. *4-Hydroxy-3-methoxymandelic acid in 24 hr. urine specimens*

| Urine | 4-Hydroxy-3-methoxymandelic acid (mg./24 hr.) |
|---|---|
| 1 | 2·5, 2·7 |
| 2 | 3·0, 3·1, 3·2 |
| 3 | 3·6, 4·0, 3·9 |
| 4 | 4·9, 5·0, 4·7 |
| 5 | 4·2, 4·6, 4·5, 4·2, 4·5 |
| 6 | 2·0, 2·4, 2·1 |
| 7 | 3·7, 3·8, 3·9 |
| 8 | 4·0, 4·0 |
| 9 | 4·2, 4·0, 4·0, 4·7 |
| 10 | 2·8, 2·5 |

difference between the curves (curve *C*) was produced by reducible material other than vanillin, which has not been identified. When the vanillin content of the extract was relatively high, as in urine from patients with phaeochromocytoma (Fig. 2*b*), curves *B* and *D* are nearly coincident; for, whereas the concentration of reducible non-vanillin substances was similar to that in extracts of normal urine, its percentage contribution to total extinction was much lower. As the absorption curve of non-vanillin reducible material was practically flat between 365 and 380 mμ, even in normal urine (six subjects), its effect on the assessment of true vanillin, and therefore of 4-hydroxy-3-methoxymandelic acid content, was minimized by the differential method of calculation.

*Recovery of 4-hydroxy-3-methoxymandelic acid from water and urine.* Replication of estimations appeared to be adequate (Table 1) and there was no great variation in the recovery of 4-hydroxy-3-methoxymandelic acid added to urine or water between concentrations of 1·25 and 100μg./ml. (Table 2). The recovery of 4-hydroxy-3-methoxymandelic acid from urine (73±6·6% S.D., 28 determinations) was similar to that from water (67±5·3% S.D., 23 determinations). Recoveries of only 40–56% of added 4-hydroxy-3-methoxymandelic acid were obtained from fresh concentrated urine specimens (8), whereas 65–75% was usually recovered from more dilute urine (5). By using 0·5 ml. of urine instead of 1·0 ml., and diluting with 1·0 ml. of water before running into the resin, the recovery of 2·5μg./ml. of added 4-hydroxy-3-methoxymandelic acid was raised from 55 to 85% and from 56 to 83% respectively in two different concentrated specimens. It was only necessary to use 0·5 ml. of urine instead of 1 ml. when the volume of the 24 hr. specimen was less than 1200 ml.

## RESULTS

The excretion of 4-hydroxy-3-methoxymandelic acid by 15 normal young adults in 24 hr. was 2·1–5·2 mg. (mean 3·9±0·7 S.D.). This range was

lower than those reported by Sandler & Ruthven (1959*a*, *b*). The present range is in better agreement with the values found by other workers (see Studnitz, 1960). This is largely accounted for by the measurement of vanillin by differential spectrophotometry which was subject to less interference from other materials than the previous colorimetric estimation and gave reproducible values (Table 1). A marked rise in 4-hydroxy-3-methoxymandelic acid output occurred in every patient with phaeochromocytoma examined. These concentrations returned to normal within 7 days after the tumour tissue had been removed (Table 3).

There is no evidence to suggest that the constituents of a general diet interfere with the assay. Ingestion of coffee, which may give falsely high readings in a qualitative test for 4-hydroxy-3-methoxymandelic acid (Gitlow, Ornstein, Mendlowitz, Khassis & Kruk, 1960), did not bring about any apparent increase of 4-hydroxy-3-methoxymandelic acid excretion in two successive 4-hourly urine samples (30 g. of 'Nescafe', one subject). Although bananas contain high concentrations of noradrenaline (Waalkes, Sjoerdsma, Creveling, Weissbach & Udenfriend, 1958) their ingestion (12 g./kg. body wt., five subjects) did not give rise to any increased 4-hydroxy-3-methoxymandelic acid excretion in eight successive hourly urine samples, when compared with a base-line value. This confirms the findings of Studnitz (1960). After 15 mg. of oral noradrenaline (one subject) no rise in 4-hydroxy-3-methoxymandelic acid output was detected in eight successive hourly samples. Even when 4-hydroxy-3-methoxymandelic acid itself was given orally (one subject) only about 35% was recovered in urine collected hourly during the next 8 hr., by which time endogenous excretion levels had been reached. This is in general agreement with the findings of Armstrong & McMillan (1959). Oral isoprenaline sulphate (40 mg., one subject) had no effect on 4-hydroxy-3-methoxymandelic acid excretion, as noted by Gitlow, Mendlowitz, Khassis, Cohen & Sha (1960).

The only drug whose ingestion so far has been found to interfere with the estimation is *p*-acetamidophenol. A subject excreted 300 $\mu$g. of 4-hydroxy-3-methoxymandelic acid in the 60 min. before ingestion of 2 g. of *p*-acetamidophenol. After dosage the value obtained was 1 mg. in 100 min. and 1·56 mg. in the next hour. The apparent increase in excretion was caused not by hydroxymethoxymandelic acid but by a substance with a different absorption curve from that of vanillin. Salicylate, which increases the extinction in the colorimetric method of Sandler & Ruthven (1959*a*, *b*), does not affect the present method. Recovery of 4-hydroxy-

Table 2. *Recovery of 4-hydroxy-3-methoxymandelic acid added to urine or water*

4-Hydroxy-3-methoxymandelic acid was added to 1·0 ml. of water, 1·0 ml. of urine (1) and 0·5 ml. of urine (2), the urines containing respectively 1·6 and 5·2 $\mu$g. of endogenous 4-hydroxy-3-methoxymandelic acid/ml.

| 4-Hydroxy-3-methoxymandelic acid added ($\mu$g./ml.) | Percentage recovery from | | |
|---|---|---|---|
| | Water | Urine 1 | Urine 2 |
| 1·25 | 77, 74, 64, 68 | 76, 84 | 66, 91 |
| 2·5 | 66, 72, 72, 72 | 74, 78 | 76, 68 |
| 5 | 70, 66, 68, 70 | 80, 78 | 74, 69 |
| 10 | 62, 65 | 77, 72 | 69, 81 |
| 20 | 65, 68, 54, 62 | 77, 78 | 73, 76 |
| 50 | 62, 65 | 66, 71 | 63, 63 |
| 100 | 70, 58, 63 | 65, 68 | 70, 70 |

Table 3. *Excretion of 4-hydroxy-3-methoxymandelic acid in patients with proven phaeochromocytoma before and after tumour removal*

| Case | 4-Hydroxy-3-methoxymandelic acid excreted (mg./24 hr.) | | Period between tumour removal and collection of urine (days) |
|---|---|---|---|
| | Pre-operative | Post-operative | |
| 1 | 14·1 | 5·6 | 14 |
| 2 | 15·5 | 4·2 | 9 |
| 3 | 10·1 | 6·9 | 4 |
| 4 | 11·6, 10·8, 12·8 | 4·6 | 29 |
| 5* | 14·6, 12·7, 11·5 | 1·1, 1·3 | 3, 7 |
| 6 | 14·3 | — | — |
| 7 | 15·5 | — | — |
| 8 | 18·6, 28·3 | — | — |
| 9 | 19·6 | — | — |
| 10 | 47·0 | — | — |
| 11 | 14·9 | — | — |
| 12 | 27·5 | — | — |
| 13 | 30·0 | — | — |
| 14 | 13·0, 20·4 | — | — |
| 15 | 23·5 | — | — |
| 16 | 17·0 | — | — |

* Infant, age 2½ years.

Table 4. *Recovery of 4-hydroxy-3-methoxymandelic acid (2·5 $\mu$g./ml.) from urines*

The number of specimens is given in parentheses.

| Description of urine | Percentage recovery |
|---|---|
| With added glucose | |
| 2% (w/v) (2) | 83; 82, 82 |
| 5% (w/v) (1) | 78, 86 |
| From patients with proteinuria | |
| 1·1 g. of protein/l. (1) | 56, 60 |
| 1·5 g. of protein/l. (1) | 67 |
| 1·9 g. of protein/l. (1) | 62, 66 |
| 2·5 g. of protein/l. (1) | 72, 72 |
| Phenylketonuria (2) | 83, 70, 70; 67, 80 |
| Maple syrup urine disease (1) | 76, 70, 78 |
| After salicylate ingestion (3) | 72, 72; 82, 78; 56, 70 |

3-methoxymandelic acid from urine after salicylate ingestion was apparently unchanged (Table 4), and its excretion appeared to remain within physiological limits (two experiments: 330 and 240 $\mu$g. in 1 hr. in control urines; 320 and 320 $\mu$g. after 100 min.; 440 and 530 $\mu$g. after 130 min.). No evidence was obtained that glucose, protein, urinary metabolites of maple syrup urine disease (Dancis & Levitz, 1960) or phenylketonuria exert any effect on the recovery of 4-hydroxy-3-methoxymandelic acid from urine (Table 4).

## SUMMARY

1. A method is described for measuring 4-hydroxy-3-methoxymandelic acid, a major metabolite of adrenaline and noradrenaline in human urine. The assay of this acid is a convenient index of catecholamine metabolism.

2. In the procedure, 4-hydroxy-3-methoxymandelic acid is absorbed on a column of anion-exchange resin and, after elution and extraction, is converted into vanillin by autoclaving with dilute acid. The vanillin is measured spectrophotometrically.

3. The accuracy and specificity of this procedure is sufficiently high to make the method suitable for following physiological fluctuations in the low concentration of 4-hydroxy-3-methoxymandelic acid (3·9 $\pm$ 0·7 mg./24 hr.) excreted by normal subjects. Mean recoveries from urine $\pm$ S.D. were 73 $\pm$ 6·6 %. Some factors affecting the recovery are discussed.

4. The value of the procedure as an aid to the clinical diagnosis of the catecholamine-secreting tumour, phaeochromocytoma, is demonstrated.

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# Teichuronic Acid: A Mucopolysaccharide Present in Wall Preparations from Vegetative Cells of *Bacillus subtilis*

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Early work (Salton, 1953, 1956; Yoshida *et al.* 1957) showed that preparations of cell-wall material made from *Bacillus subtilis* are likely to be more complicated than those from some other Gram-positive organisms such as *Micrococcus lysodeikticus*, *Staphylococcus aureus* or *Bacillus megaterium*. Preparations from these last-named organisms revealed, after acid hydrolysis, the presence of only three or four amino acids as major components together with two amino sugars and in some instances one or two hexoses (Salton, 1953; Cummins & Harris, 1956; Salton & Milhaud, 1959). In some strains of the two coccal species up to 80–90 % of the wall is made from four amino acids

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and two amino sugars combined as mucopeptides (Perkins & Rogers, 1959; Mandelstam & Rogers, 1959). In contrast, preparations from *B. subtilis* yielded nine amino acids as well as two amino sugars and glucose (Salton, 1956). Moreover, wall preparations from organisms such as *M. lysodeikticus* and *B. megaterium* are rendered almost completely soluble by egg-white lysozyme, whereas preparations from *B. subtilis* are made only partially soluble, leaving material which is said to retain the form of the wall (Salton, 1956). These observations would suggest the presence of at least two components in the wall: one sensitive to and the other resistant to lysozyme. Further work on this problem is presented below.

Also present in wall preparations from *B. subtilis* are the teichoic acids. The discovery and investigation of these substances has been described by Baddiley (1959) and a detailed investigation of teichoic acid from *B. subtilis* published (Armstrong, Baddiley & Buchanan, 1960). In this organism the teichoic acid is a polymer of ribitol phosphate with glucose and alanine attached, the latter in ester linkage.

Teichoic acid can be extracted from the wall preparations by cold trichloroacetic acid solutions, and it was during such treatment we observed that the extracts also contained considerable amounts of a polymer containing a *N*-acetylhexosamine. The extraction of this polymer from the walls led to a reduction in the otherwise high ratio of hexosamine to 3-carboxyethylglucosamine (muramic acid) present in acid hydrolysates of the preparations. Examination of the substance in the extracts which contained hexosamine revealed that it was composed entirely of *N*-acetylgalactosamine and glucuronic acid, thus resembling chondroitin isolated from bovine cornea (Davidson & Meyer, 1955). Examination of the mode of linkage of the hexosamine and hexuronic acid has suggested that the material from *B. subtilis*, which we propose to call teichuronic acid, has in fact $\alpha$-glycosidic linkages instead of the $\beta$-linkages in chondroitin. A brief preliminary account of this work has already appeared (Janczura, Perkins & Rogers, 1960).

## MATERIALS AND METHODS

*Organism.* The strain of *Bacillus subtilis* used was N.C.T.C. 6346.

*Growth medium.* A casein acid-hydrolysate medium of the following composition was used: Casamino acids (Difco), 10 g.; $KH_2PO_4$, 2·72 g.; water to 900 ml. The pH was adjusted to 7·2 with NaOH solution. This medium was sterilized by autoclaving at 120° for 20 min. and then supplemented by 100 ml. of 17 mM-$MgSO_4$, 1·0 ml. of a solution of trace metals, containing 0·1 mg. each of $ZnSO_4,7H_2O$, $CoCl_2,6H_2O$, ammonium molybdate, $MnCl_2,4H_2O$ and $CuSO_4,5H_2O$/l., and 1 ml. of a solution containing 0·156 g. of $FeSO_4,(NH_4)_2SO_4,6H_2O$ and 0·168 g. of citric acid/100 ml. of water.

*Inoculation of medium and growth of cultures.* A portion (100 ml.) of the above-described medium was inoculated with 0·25 ml. of a suspension of spores of the organism (containing $3 \times 10^8$ viable organisms/ml.) which had previously been incubated at 35° overnight. The small culture was incubated in a 1 l. flask overnight at 35° and shaken to increase aeration. It was then added to the bulk of the above-described medium (4·5 l.) contained in five 5 l. flasks which were again shaken at 35° overnight.

*Preparation of suspensions of micro-organisms and cell walls.* The cultures, after overnight incubation, were centrifuged at 1000*g* for 20 min. and the deposited organisms washed with 800 ml. of ice-cold 0·9% NaCl soln. About 10 g. dry wt. of cells was obtained from a 5 l. batch of culture.

It is necessary in preparing cell walls from many organisms to ensure that lytic enzymes are inactivated, otherwise the yield of cell wall obtained is much reduced and the composition of the residue may not be representative. In the earlier part of this work the enzymes were inactivated by treating the cell suspensions with trichloroacetic acid. Later it was found that very satisfactory yields of wall could be obtained by heating water suspensions of the crude preparations to 100° for 20 min. In the early work treatment with trichloroacetic acid was usually at 0°, but in some instances the cells were first heated with acid for 15 min. at 90°. After the treatment with trichloroacetic acid the cells at a density of 10 mg./ml. were treated with crystalline ribonuclease and trypsin (1 mg./ml.) in 0·05M-buffer, pH 7·6, at 35° in the presence of toluene, followed, after the preparation had been washed, by crystalline pepsin (1 mg./ml.) in 0·05 N-HCl; both treatments were continued until the absorption of ultraviolet light at a wavelength of 280 m$\mu$ of the insoluble cell-wall material fell to a minimum. In some other experiments the cells, after treatment with either hot or cold trichloroacetic acid, were shaken with small glass beads of 0·1 mm. diameter on the Mickle (1948) disintegrator for varying times and the insoluble residue was isolated by centrifuging for 10 min. at 10 000*g*. The residue was resuspended in water and unbroken cells were removed by centrifuging at 1000*g* for 10 min. The cell-wall material was again treated with the proteolytic enzymes as described above. Finally all the cell-wall preparations were washed in acetone and ether and dried *in vacuo*. The purpose of the above variations in technique was: (1) to stop the action of the active cell-wall-hydrolysing enzymes present in *B. subtilis*; (2) to attempt to remove the material containing a wide range of amino acids from the mucopeptide and thus to obtain wall preparations resembling those from other organisms, such as the cocci, which consist of mucopeptides containing only four or five amino acids. However, the composition of the material obtained by the types of treatment outlined above was qualitatively always the same in its amino acid and amino sugar composition. Experiments in which the quantitative composition of the wall material was measured showed that the relative amounts of the various amino acids were also approximately constant. As will be shown later, the quantitative amino sugar composition was dependent upon previous treatment.

In later experiments it was found that cell-wall preparations of the same composition were obtained without the use of trichloroacetic acid by the Cummins & Harris (1956) technique, provided that the following modifications were introduced: (1) all procedures between the end of incubating the cultures and the differential centrifuging of the cell walls were performed at 0–4°; (2) the suspension of cell walls once obtained was raised to 100° for 20 min. before treatment with enzymes, as has previously been described for the preparation of mucopeptide from *S. aureus* (Mandelstam & Rogers, 1959); (3) treatment with trypsin and ribonuclease was continued overnight (i.e. 16 hr.), but when teichuronic acid was to be isolated the treatment with pepsin used by Cummins & Harris (1956) was omitted and the preparations were not dried. When the hot-water method was used the isolated wall material accounted for 20–25% of the dry weight of the organism.

*Hyaluronidase and mucopolysaccharides.* The bovine testicular hyaluronidase was Rondase (Evans Medical Supplies, London) and the staphylococcal hyaluronidase was that present in supernatants from cultures of *Staphylococcus aureus* strain 524 SC incubated for 18 hr. at 35° under the conditions previously described (Rogers, 1953). The hyaluronic acid was kindly given to us by Dr J. E. Scott, and the chondroitin sulphate A used for preparation of chondroitin was from Dr Helen Muir, to whom we are grateful. Chondroitin was prepared from chondroitin sulphate A by the method of Kantor & Schubert (1957). The product obtained contained less than 0·1% of sulphur.

*Lysozyme digestion of walls.* Unless otherwise stated in the text, the walls were suspended at 1 mg./ml. in 0·1M-ammonium acetate solution adjusted to pH 6·5. This suspension was incubated at 35° and crystalline egg-white lysozyme (Armour Laboratories) equivalent to 50$\mu$g./ml. was added.

*Infrared spectra.* Infrared absorption was measured in compressed KCl disks on a Perkin–Elmer double-beam instrument with rock-salt prism.

### *Analytical methods*

*Hexosamine.* This was estimated after hydrolysis for 4 hr. at 100° with 4N-HCl by the method of Rondle & Morgan (1955), after separation when necessary from 3-carboxyethylglucosamine as described by Perkins & Rogers (1959).

*Hexuronic acid.* The colorimetric estimation of Dische (1947) was used.

*Phosphorus.* This was estimated by the technique of Allen (1940).

*Amino acids.* These were estimated after paper chromatography by a modification of the method of Kay, Harris & Entenman (1956).

*Paper electrophoresis.* The apparatus of Kunkel (1954) was used with a potential difference of 15 v/cm. applied for 2–3 hr. The following buffers were used: collidine–acetic acid, pH 6·5 (Newton & Abraham, 1954); 2 N-acetic acid.

*Paper chromatography.* Descending chromatography on Whatman no. 3 paper was used throughout the work. Two solvent systems were used: (*a*) butan-1-ol–acetic acid–water (6·3:1·0:2·7, by vol.); (*b*) butan-1-ol–pyridine–water (6:4:3, by vol.).

## RESULTS

### *Mucopeptide and protein content of walls*

Preliminary experiments showed that when the wall preparations were hydrolysed for 16 hr. with 6N-HCl at 100° and the hydrolysate was examined by two-dimensional chromatography, 15 or 16 ninhydrin-positive areas could be distinguished. The relative positions of those areas which could be clearly identified corresponded to those for alanine, aspartic acid, glutamic acid, glycine, $\alpha\varepsilon$-diaminopimelic acid, valine, leucine or isoleucine or both, threonine, serine, lysine, phenylalanine, proline, glucosamine and muramic acid. Of the amino acids, alanine, glutamic acid, glycine, aspartic acid and the leucine–isoleucine pair were in greatest molar concentration. Next came $\alpha\varepsilon$-diaminopimelic acid, valine and phenylalanine at about one-third to one-quarter of the concentration, and there were smaller amounts of serine, lysine, tyrosine and proline.

Examination of the effect of egg-white lysozyme upon these preparations confirmed other work (Salton, 1956), showing that the turbidity of the suspensions could be reduced only to about 30–40% of the initial value, and that further incubation or addition of enzyme caused no further digestion of the cell walls. The immediate deduction, however, that this decrease in turbidity indicates that 30–40% of the wall preparation was left unhydrolysed is not supported by isolation of the residue. When the suspension remaining after 24 hr. incubation with lysozyme in the presence of toluene was centrifuged at 10 000*g* for 10 min. a small deposit was obtained and a clear supernatant fluid. The deposit was washed once with water and then transferred to a tared tube with a minimum quantity of water, mixed with ethanol and centrifuged at 1000*g* for 20 min. Finally it was washed with acetone and ether and dried *in vacuo*. In two different experiments the weight of this deposit represented 7·2% and 11·1% of the weight of the cell walls. This figure agrees well with that of 7% reported by Salton & Marshall (1959) as 'insoluble wall residue'.

Examination was then made of the composition of the material dissolved by the action of lysozyme and of the residue. The supernatant obtained after digestion of the wall material with lysozyme was dried and the ammonium acetate removed *in vacuo*. A weighed amount of both the residue and the soluble material was then hydrolysed with 6N-HCl at 105° for 16 hr. The dried hydrolysates after solution in water were examined for the distribution of ninhydrin-staining areas by chromatography in two directions. Table 1 shows the results obtained compared with those for the whole wall. It will be seen that the only amino acids occurring in

large amounts in the part of the wall material rendered soluble by lysozyme were $\alpha\epsilon$-diaminopimelic acid, glutamic acid and alanine. A very small amount of glycine equivalent to about 0·04 times the amount of alanine was constantly present in a large number of experiments. Its significance is unknown but a similar small amount of glycine in whole wall material from a different organism has already been reported (Rogers & Perkins, 1959). It is notable that all the diaminopimelic acid and all of both the amino sugars are found in the soluble part. Thus it seems reasonable to suppose that the mucopeptide in this organism contains as major constituents alanine, $\alpha\epsilon$-diaminopimelic acid, glutamic acid, muramic acid and glucosamine. The residue to which this is attached contains at least 12 amino acids and probably has a normal protein composition. This result is to be contrasted with that obtained by Salton & Marshall (1959), who reported the presence of only traces of four amino acids other than alanine, glutamic acid and diaminopimelic acid in cell-wall preparations from *B. subtilis*. The protein is resistant to both trypsin and pepsin whilst still associated with the mucopeptide, but no work has been done to see whether it is resistant after removal of the mucopeptide by lysozyme.

Table 1. *Composition of the residue and material dissolved from the cell-wall preparations from* Bacillus subtilis *by lysozyme*

The + signs give a semi-quantitative indication of the amounts of the various substances. For comparison the results from an examination of the untreated wall material are also included. tr., Trace; —, Not known.

| Substance | Whole wall material | Wall material after lysozyme treatment | |
|---|---|---|---|
| | | Soluble | Insoluble |
| Alanine | + + + | + + + | + + |
| Aspartic acid | + | 0 | + + + |
| Diaminopimelic acid | + + + | + + + | 0 |
| Glutamic acid | + + + | + + + | + + + |
| Glycine | + | + | + + + |
| Leucine } Isoleucine } | + | 0 | + |
| Lysine | tr. | 0 | tr. |
| Methionine | + | — | — |
| Phenylalanine | + | 0 | + + |
| Proline | + | — | tr. |
| Serine | + | 0 | tr. |
| Threonine | + | 0 | + + |
| Tyrosine | + | 0 | tr. |
| Valine | + | 0 | + |
| Glucosamine | + + | + + | 0 |
| Muramic acid | + | + | 0 |

### *Recognition of the presence of a hexosamine-containing polymer apart from mucopeptide*

During the course of analysis of a number of cell-wall preparations a very considerable variation in the molar ratio of unsubstituted hexosamine to 3-carboxyethylglucosamine was noted. This ratio was sometimes as great as 3 or 4 to 1 and sometimes small enough to indicate nearly equimolar amounts of the two substances. Further examination of this situation showed that the lowest ratios were correlated with the use of hot 5% trichloroacetic acid (see Methods section) during preparation of the walls, whereas material with the highest ratio resulted from the method in which there was no treatment with trichloroacetic acid. It therefore seemed possible that trichloroacetic acid was removing a substance containing unsubstituted hexosamine rather than 3-carboxyethylglucosamine. To test this possibility walls prepared after treatment of the crude product with hot water alone (see Methods section) were subjected to various treatments with 5% (w/v) trichloroacetic acid. As will be seen from Table 2, the molar ratio of the hexosamine to 3-carboxyethylglucosamine was 3·6:1 in an untreated wall preparation and by treatment with hot trichloroacetic acid the ratio could be reduced to approximately 1:1.

Table 2. *Treatment of cell-wall preparation with trichloroacetic acid*

The cell walls were first prepared by the method in which the lytic enzymes were inactivated by raising the temperature of a suspension in water to 100° for 20 min. In Expt. 2, walls already extracted three times with 5% trichloroacetic acid at 37° for 24 hr. were further extracted three times with fresh portions of 5% trichloroacetic acid, each time for 15 min. at 90°.

| Expt. | Treatment | Temperature | Time | μmole/mg. Hexosamine (A) | μmole/mg. 3-Carboxyethylhexosamine (B) | Ratio (A/B) |
|---|---|---|---|---|---|---|
| 1 | None | — | — | 0·93 | 0·26 | 3·6 |
| | 5% Trichloroacetic acid | 20° | 18 hr. | 0·84 | 0·31 | 2·7 |
| | | 90 | 15 min. | 0·63 | 0·31 | 2·0 |
| | | 90 | 60 min. | 0·65 | 0·39 | 1·7 |
| 2 | 5% Trichloroacetic acid | 90 | 15 min. (three times repeated) | 0·81 | 0·70 | 1·2 |

*Examination of trichloroacetic acid extracts*

In order to examine the nature of the substances extracted by trichloroacetic acid solution, 200 mg. of cell-wall preparation (made with hot-water treatment alone to inactivate the lytic enzymes) was mixed with 10 ml. of 5 % (w/v) trichloroacetic acid. This suspension was shaken for 16 hr. at room temperature, the cell-wall material was then deposited by centrifuging and re-extracted with trichloroacetic acid twice more under the same conditions. The extracts were combined and four to five times their volume of acetone was added. A gluey precipitate (about 8 mg.) was obtained which was dried with ether. This material was dissolved in 2·0 ml. of water and 0·7 ml. applied as a band to Whatman no. 3 paper and subjected to electrophoresis with 2M-acetic acid as buffer. The applied potential was 15 v/cm. and the time 3 hr. After drying, a longitudinal 1 cm. strip of paper was cut off and stained according to the method of Rydon & Smith (1952). Two anionic zones appeared, the slower (A) having moved 6 cm. and the faster (B) 14 cm.; the region of the origin also showed some staining. The areas corresponding to the two zones A and B in the remainder of the paper which had not been stained were separately eluted with water and the eluates dried. The resulting materials will be temporarily referred to as A and B.

Both materials were examined: (1) hexosamine was estimated after hydrolysis for 4 hr. at 100° with 4N-HCl; (2) ninhydrin-positive areas were noted after chromatography of the hydrolysates in two dimensions; (3) a hydrolysate made with 2N-HCl acting for 2 hr. at 100° was examined for hexose after chromatography in one direction with butanol–acetic acid–water as solvent; (4) total phosphorus was estimated; (5) hexuronic acid was estimated on the unhydrolysed material.

No significant amounts of amino acids were present in either of the materials, except a trace of alanine in substance B. Hexosamine and hexuronic acid tests were positive in material A. In material B there was phosphorus and glucose but no hexosamine or hexuronic acid. Material B was likely to be teichoic acid, which had already been isolated from wall preparations of *B. subtilis* (Armstrong *et al.* 1960) by extraction with trichloroacetic acid. Further examination of several preparations showed that the molar ratio of phosphorus to glucose varied from 1·2 to 0·8 and that solutions of the material showed no specific absorption of ultraviolet in the region of 260 mμ. The almost complete absence of alanine from substance B seemed at first anomalous since the teichoic acid contains about 1 mole of this amino acid/mole of phosphorus. Further consideration of the great alkali-lability of the bond by which alanine is linked to teichoic acids (Armstrong *et al.* 1960), however, suggested the probability that it had been removed during the treatment of the wall preparations with trypsin and ribonuclease at pH 7·6. It was considered that substance B was teichoic acid and it was not examined further.

Substance A yielded only hexosamine and hexuronic acid on hydrolysis. It is this substance we propose to call teichuronic acid.

*Isolation and properties of teichuronic acid*

The following method was adopted as routine for the preparation of teichuronic acid. It is not claimed that this is the best method and indeed it is likely to reduce the size of the molecule considerably from that of the substance in the cell-wall material itself. It does, however, yield a reasonable amount of mucopolysaccharide in a satisfactory state of purity.

Cell-wall material was prepared from the cells obtained from 5 l. of culture by the method which involves heating a water suspension to inactivate the lytic enzymes (cf. Methods section). About 1·2 g. dry wt. of material was used. It was suspended without first drying in 150 ml. of 5 % (w/v) trichloroacetic acid and left at room temperature overnight (i.e. about 15–18° for 16 hr.). This removes relatively little teichuronic acid but a lot of the teichoic acid and the first extract was discarded. The walls were then resuspended in a further 150 ml. of 5 % trichloroacetic acid and left at 35° for 16 hr. This extraction at 35° was repeated three more times. The combined extracts (two to four) were then extracted three times with an equal volume of ether to remove the trichloroacetic acid, and the pH was adjusted to 7·0 by the addition of a few drops of N-NaOH. The volume of the extracts was then reduced to 100 ml. by evaporation at a pressure of 15 mm. Hg, the temperature of the outside bath being not greater than 45°. The pH of the solution was then adjusted to 3·2 with 2N-$H_2SO_4$ (about 0·15 ml. was required) and a 1 % (w/v) solution of cetylpyridinium bromide added until no further precipitation occurred. The suspension was then warmed to 30° to agglutinate it and the precipitate was removed by centrifuging, washed with 0·1 % cetylpyridinium bromide and dissolved in 20 ml. of 0·3M-$Na_2SO_4$. A very slight amount of material which failed to dissolve was deposited by centrifuging and discarded. The supernatant was placed in a tube of cellophan and dialysed against 0·05M-$Na_2SO_4$ for 16 hr. with stirring. The suspension was then centrifuged and a very oily precipitate formed. This was dissolved in 20 ml. of 0·2M-$Na_2SO_4$ and 40 ml. of ethanol added. The thick precipitate of $Na_2SO_4$ crystals

was removed. A further 40 ml. of ethanol was then added and on the addition of a few crystals of potassium acetate a further precipitate formed. This was deposited by centrifuging, washed twice with ethanol, once with ether and dried *in vacuo* (yield 81 mg.). Various batches of material had the analyses indicated in Table 3. Neither phosphorus nor sulphur could be detected.

The material as isolated consisted of a white powder readily soluble in water to form somewhat viscous solutions. When dialysed against water in Visking cellophan tubing none of the material passed through the membrane. When subjected to electrophoresis on paper at pH 2·4 it moved further towards the anode than hyaluronic acid. At pH 6·5 it moved anionically more rapidly than hyaluronic acid and less than chondroitin sulphate. It remained at the origin at pH 2·0. These results probably indicate that the effective negative charge on the teichuronic acid molecule is greater than that on hyaluronic acid but less than that on chondroitin sulphate. In assessing the value of the evidence for this, however, it should be borne in mind that from the method of isolation the molecular size of teichuronic acid might be expected to be considerably smaller than that of the other two mucopolysaccharides, and this may lead to alterations in the rate of movement on paper.

### *Behaviour of teichuronic acid on a cellulose ion-exchange material*

The homogeneity of the preparations of teichuronic acid was further demonstrated by chromatography on a column of diethylaminoethylcellulose. The column was prepared as described by Rogers & Perkins (1960) and equilibrated with 0·01 M-ammonium acetate buffer, pH 5·0. It was eluted with a gradient of the same buffer, starting with 1 l. of 0·01 M-ammonium acetate in the mixing flask and using concentrations of firstly 0·5 M and secondly 5 M in the reservoir. Fractions (2·5 ml.) were collected and samples were taken for estimation of uronic acid. Fig. 1 shows that a single sharp peak was obtained. This peak also contained the hexosamine.

*Oxidation with sodium periodate.* Jeanloz & Forchielli (1951) studied the periodate oxidation of hyaluronic acid and showed that at pH 4·5 and 5° over-oxidation was prevented. Under these conditions hyaluronic acid consumed about 0·3 mole of oxidant/repeating unit in 25 hr. To see whether teichuronic acid resembled hyaluronic acid in general structure, and in particular whether it had an excessive proportion of end groups characteristic of a branched-chain polymer, we oxidized each of these mucopolysaccharides with periodic acid under the conditions described above, and measured the disappearance of periodate. The results (Table 4) show that teichuronic acid and hyaluronic acid behave similarly toward periodate oxidation. The slightly greater over-oxidation of teichuronic acid may indicate that the degree of polymerization of the sample as isolated was less than that of the hyaluronic acid used. It is known (see discussion by Rogers, 1961) that the small periodate consumption observed with hyaluronic acid does not preclude the presence of vicinal OH

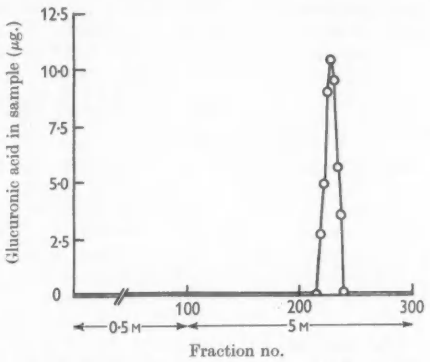


Fig. 1. Gradient chromatogram on *NN*-diethylaminoethylcellulose column. Gradient was made by running (*a*) 0·5 M- and (*b*) 5 M-ammonium acetate into 1 l. of 0·01 M-ammonium acetate in a closed vessel. Both buffers were pH 5·0. Teichuronic acid was estimated by measuring the amount of glucuronic acid by the method of Dische (1947).

Table 3. *Analytical data for different batches of teichuronic acid*

Calculated values for a polymer of *N*-acetylgalactosamine and glucuronic acid are: for the free acid, 47·2% of galactosamine, 51·2% of glucuronic acid; for the potassium salt, 42·9% of galactosamine, 46·5% of glucuronic acid.

| Sample | Batch | Galactosamine (%) | Glucuronic acid (%) | Molar ratio (glucuronic acid/galactosamine) |
|---|---|---|---|---|
| Potassium salt | 1 | 33·2 | 38·4 | 1·07 |
| | 2 | 32·2 | 38·1 | 1·09 |
| Free acid | — | 43·9 | 44·8 | 0·93 |
| Material from electrophoresis strip | $(A_2)$ | — | — | 1·04 |
| | $(A_4)$ | — | — | 1·01 |

groups and therefore of substitution on positions other than C-3 on the hexose molecules in the polysaccharide, since a C-4 linkage has now been shown to occur on the glucuronic acid moiety of hyaluronic acid (Meyer, 1957, 1958). The same reasoning may apply to teichuronic acid.

*Action of hyaluronidases.* Since teichuronic acid resembled hyaluronic acid and chondroitin in many respects it was interesting to see whether it would be attacked by hyaluronidases. As there was also the possibility, mentioned below, that teichuronic acid might contain α- rather than β-glycosidic linkages, it might have been expected that enzymes which normally attack β-linked mucopolysaccharides would be without effect upon it.

Solutions of the various substrates were prepared at such a concentration that they yielded a conveniently dense precipitate with serum reagent at pH 4·2 (Tolksdorf, McCready, McCullagh & Schwenk, 1949). The substrates were incubated at pH 6·0 and 37° with suitable dilutions of either testicular hyaluronidase (stock solution 1500 i.u./ml.) or the supernatant from an overnight culture of *Staphylococcus aureus* 524 SC, which is known to contain much hyaluronidase (Rogers, 1953). Incubation was continued either for 10 min. or, if little action had occurred, overnight. The final turbidity obtained upon addition of acetate buffer, pH 4·2, and serum reagent was read on a Spekker colorimeter with a neutral Ilford filter H 508. In addition to hyaluronic acid and teichuronic acid, chondroitin which has the same gross chemical composition as teichuronic acid but is known to be β-linked was also used as a substrate. The results are given in Table 5. Testicular hyaluronidase attacked hyaluronic acid with extreme readiness and chondroitin at about one-twentieth of the rate, but was virtually without effect on teichuronic acid. Staphylococcal filtrates behaved similarly with respect to the first two substrates, but also showed some action on teichuronic acid. The inability of testicular hyaluronidase to attack teichuronic acid offers some support for the idea that the configuration of its glycosidic linkages is different. It is not clear whether the attack by unpurified staphylococcal filtrates can be attributed to the hyaluronidase which they are known to contain, or to some other enzyme.

Table 4. *Periodate oxidation of hyaluronic acid and teichuronic acid*

Oxidation was performed in 0·1 M-acetate buffer, pH 4·5, at 5°.

| Substance | Time (hr.) | Periodate consumed (mole/repeating unit) |
|---|---|---|
| Teichuronic acid | 3·5 | 0·20 |
| | 5·5 | 0·28 |
| | 22 | 0·56 |
| Hyaluronic acid | 3·5 | 0·24 |
| | 5·5 | 0·26 |
| | 22 | 0·42 |

*Components of teichuronic acid*

*Nature of the amino sugar.* A hydrolysate of teichuronic acid was prepared by heating with 4 N-HCl for 4 hr. at 100° and, after the acid had been removed *in vacuo*, the hexosamine was separated by paper chromatography with the solvent system butanol–pyridine–water. It produced a single ninhydrin-positive spot with $R_F$ slightly less than that of glucosamine. The eluted hexosamine was then oxidized with ninhydrin according to the technique of Stoffyn & Jeanloz (1954). The pentoses formed were separated by zone electrophoresis in 0·025 M-borate buffer, pH 9·0. The only pentose which could be recognized by this technique was lyxose; no arabinose was formed. This clearly indicated that the original hexosamine was not glucosamine, but might be galactosamine or talosamine. The hexosamine was

Table 5. *Action of hyaluronidase on teichuronic acid and chondroitin*

Enzyme action was measured by the decrease in turbidity obtained after addition of serum reagent at pH 4·2. Stock enzyme solutions were (*a*) testicular hyaluronidase (1500 i.u./ml.) and (*b*) supernatant from an overnight culture of *Staphylococcus aureus* 524 SC. The dilutions of the enzyme were made in 0·4% gelatin.

| Substrate | Enzyme | Dilution | Incubation time | Decrease in turbidity (%) |
|---|---|---|---|---|
| Hyaluronic acid | Testicular | 40 | 10 min. | 42 |
| | Bacterial | 100 | 10 min. | 84 |
| Teichuronic acid | Testicular | 2 | 10 min. | 0 |
| | Testicular | 2 | 24 hr. | 13 |
| | Bacterial | 4 | 10 min. | — |
| | Bacterial | 4 | 24 hr. | 37 |
| Chondroitin | Testicular | 2 | 10 min. | 36 |
| | Testicular | 2 | 24 hr. | 100 |
| | Bacterial | 4 | 10 min. | 16 |
| | Bacterial | 4 | 24 hr. | 53 |

then *N*-acetylated under conditions similar to those described by Cardini & Leloir (1957), and run on a paper chromatogram with butanol–pyridine–water as solvent. The single spot giving the purple colour characteristic of *N*-acetylated hexosamines (Partridge, 1948) corresponded in position with authentic *N*-acetylgalactosamine, and was clearly distinguished from the glucosamine and talosamine derivatives (Crumpton, 1959). Since the *N*-acetylated material gave the Morgan–Elson reaction for *N*-acetylhexosamines, it could not have been the 2:4-diacetamidodideoxyhexose isolated from *B. subtilis* by Sharon & Jeanloz (1958, 1960), because their material, in common with other 2-acetamidohexoses substituted in the C-4 position, did not give this reaction (Kuhn, Gauhe & Baer, 1954).

*Hexuronic acid.* As mentioned above, teichuronic acid gave a positive carbazole reaction (Dische, 1947) for hexuronic acid. It is known that the various hexuronic acids give very different ratios of colour intensity in the carbazole and orcinol reactions (Khym & Doherty, 1952), only glucuronic acid and galacturonic acid giving ratios of approximately 1·0 (Hoffman, Linker & Meyer, 1956). Iduronic acid, guluronic acid and mannuronic acid give carbazole/orcinol ratios of 0·22, 0·30 and 0·13 respectively. It was found that a sample of teichuronic acid gave a carbazole/orcinol ratio of 1·1, suggesting that either glucuronic acid or galacturonic acid was present. To distinguish between these possibilities, samples of teichuronic acid were hydrolysed in 0·5N-HCl at 100° for 3 hr. and the hydrolysates were run on paper chromatograms in butanol–acetic acid–water and ethyl acetate–acetic acid–water (3:1:3, by vol.) (Rao, Beri & Rao 1951). The hydrolysates produced spots corresponding only to glucuronic acid and its lactone and not to galacturonic acid, which in any case does not form a lactone. Hence we concluded that the hexuronic acid component of teichuronic acid was glucuronic acid.

### *Nature of the glycosidic bonds*

The material isolated from the trichloroacetic acid extracts, either by paper electrophoresis as described earlier in this paper or by precipitation by cetylpyridinium bromide, did not diffuse through cellophan membranes and behaved generally like a substance of relatively high molecular weight (e.g. concentrated solutions showed detectable viscosity). It therefore seemed likely from the analyses that teichuronic acid was a polymer made up of equimolar amounts of *N*-acetylgalactosamine and glucuronic acid. The position and configuration of the supposedly glycosidic bonds were, of course, unknown.

*Optical rotation.* As can be seen from Table 3, the amounts of *N*-acetylgalactosamine and glucuronic acid present in the various preparations amounted to about 80% of the dry weight of the material. In order to calculate the specific optical rotation it was assumed that the remaining 20% of the material represented ash and water. On this basis $[\alpha]_D^{23}$ had a value of $+38°$ to $+40°$. The positive direction of rotation is opposite from that of chondroitin (Davidson & Meyer, 1955; Kantor & Schubert, 1957) or that of hyaluronic acid, both of which have a high negative rotation. This result suggested to us that in contrast to these two polysaccharides the configuration of one or both types of glycosidic bond in teichuronic acid might be $\alpha$ instead of $\beta$. Further work, however, was clearly necessary to establish this.

*Acid hydrolysis.* When hyaluronic acid or chondroitin sulphate is hydrolysed with dilute acids disaccharides with the structure 3-*O*-$\beta$-glucuronosyl-2-deoxyaminohexose can be isolated. Weissman, Rapport, Linker & Meyer (1953) hydrolysed hyaluronic acid with 0·5N-$H_2SO_4$ at 100° for 3 hr. and obtained 20–30% yield of crystalline hyalobiuronic acid. We treated a sample of teichuronic acid in the same way, hydrolysing for 20, 40, 60 and 90 min. Chromatographic examination of the products showed only unchanged material and progressively increasing amounts of free galactosamine, together with free *N*-acetylhexosamine in the early samples. The *N*-acetylhexosamine ran in the same position as an authentic sample of *N*-acetylated galactosamine. There was no sign of any appreciable yield of disaccharide, in contrast with the results of Weissman *et al.* (1953) with hyaluronic acid. This result suggests the presence in teichuronic acid of the more acid-labile $\alpha$-linkage.

Further partial hydrolyses were performed in an attempt to identify the point of attachment of the glucuronic acid residues to those of *N*-acetylgalactosamine. As mentioned above, the yields of disaccharide were very small, but nevertheless it still proved possible to apply the technique of Cifonelli & Dorfman (1958). In this method samples of mucopolysaccharide are partially hydrolysed in HCl and the colour produced by the hydrolysates in the Elson–Morgan reaction is examined. A low ratio for the relative light-absorption at 540 m$\mu$ and at 510 m$\mu$ indicates the presence of substituents on the C-3 position of the hexosamine. Glucosamine hydrochloride gives a value of 1·38.

Samples of teichuronic acid were hydrolysed in 2N-HCl at 100° for various periods and the hydrolysates were examined in the Elson–Morgan reaction (Rondle & Morgan, 1955). The comparative values for light-absorption at 540 m$\mu$ and 510 m$\mu$ are given in Table 6, together with the results of Cifonelli & Dorfman (1958) on hyaluronic acid and chondroitin sulphate A. The result for

15 min. hydrolysis makes it probable that the glucuronic acid is linked to the galactosamine at the C-3 position. After standing overnight, the coloured solution from the sample hydrolysed for 15 min. showed an increase in light-absorption (also characteristic of hexosamines substituted at C-3) (Crumpton, 1959) and the absorption curve shown in Fig. 2 was obtained. Substituents at C-3, C-4 and C-6 give absorption maxima in the Elson–Morgan reaction at 510, 530 and 540 m$\mu$ respectively. This evidence points to the presence of a 3-glucuronosyl-*N*-acetylgalactosamine unit in teichuronic acid.

Table 6. *Relative absorption at 540 and 510 m$\mu$ in Elson–Morgan reaction given by partial hydrolysates of mucopolysaccharides*

2 N-HCl at 100° was used for the hydrolysis. Extinctions were read immediately after development of the colour in the Rondle & Morgan (1955) reaction.

| Time of hydrolysis | $E_{540\ m\mu}/E_{510\ m\mu}$ Hyaluronic* acid | Chondroitin* sulphate A | Teichuronic acid |
|---|---|---|---|
| 15 min. | — | — | 0·77 |
| 30 min. | 0·37 | 0·45 | 0·95 |
| 60 min. | 0·44 | 0·48 | 0·91 |
| 120 min. | 0·72 | 0·71 | 0·98 |
| 16 hr. | 1·15 | 1·20 | — |

* Results obtained by Cifonelli & Dorfman (1958).

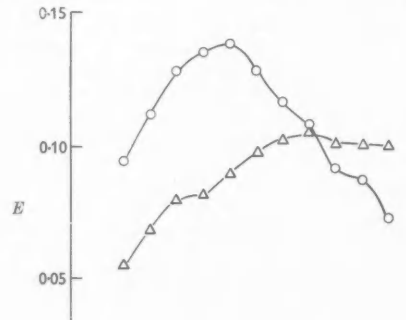


Fig. 2. Absorption curves in Elson–Morgan reaction. The 15 min. partial hydrolysate of teichuronic acid (see Table 6) was treated by the Rondle & Morgan (1955) procedure. The absorption curve was prepared after the final solution had stood overnight. ○, Teichuronic acid hydrolysate; △, glucosamine hydrochloride.

*Alkaline hydrolysis.* It was found that samples of teichuronic acid, although diffusible and clearly macromolecular, gave exceptionally high values for free *N*-acetylhexosamine under the conditions of the Morgan–Elson reaction, as modified by Reissig, Strominger & Leloir (1955). For instance, after heating for 15 min. at 100° in borate buffer, pH 9·0, a sample of teichuronic acid gave a reaction with Ehrlich's reagent corresponding to the presence of 22 % of its weight as *N*-acetylgalactosamine, compared with the theoretical total content of 58 %. A sample of hyaluronic acid in a parallel experiment gave no colour at all. Once again it appeared that the glycosidic links in teichuronic acid were of exceptional lability. Some confirmation that breakdown was taking place under the alkaline conditions was obtained as follows. A sample of teichuronic acid was heated for 4 min. in a sealed ampoule at 100° with triethylamine (Perkins, 1960). Chromatography of the product compared with untreated material showed that the alkaline treatment had liberated much of the same Morgan–Elson chromogen given by free *N*-acetylhexosamines, observable as a spot with $R_F$ 0·60 in butanol–pyridine–water. The untreated teichuronic acid had not moved from the origin.

*Infrared spectroscopy.* Examination of infrared spectra provided further evidence for the configuration of the glycosidic linkages in teichuronic acid. Barker, Bourne, Stacey & Whiffen (1954) showed that among many glycosides and polysaccharides, including some amino sugar glycosides, α-linked compounds were characterized by an absorption maximum at about 844 cm.$^{-1}$, whereas a β-link produced a peak at about 890 cm.$^{-1}$, though this latter peak might easily be obscured by others. We compared the infrared spectrum of a sample of chondroitin with that of teichuronic acid. Both substances were used as the free acid.

The results are shown in Fig. 3. A peak at 845–865 cm.$^{-1}$, present in the spectrum of teichuronic acid and absent (or shifted to the region 875–900 cm.$^{-1}$) in that of chondroitin, suggests that α-glycosidic links occur in teichuronic acid.

## DISCUSSION

In confirmation of the results obtained by Salton (1953, 1956) and Yoshida *et al.* (1957) we find that the cell walls of the strain of *B. subtilis* which we have examined contain materials composed of more amino acids than do similar preparations from other Gram-positive organisms. Irrespective of the method of preparation of the wall material it contained about 7–10 % of protein material which was not made soluble either by lysozyme or by prolonged incubation with trypsin or pepsin. The amino acid content of this material appeared in no

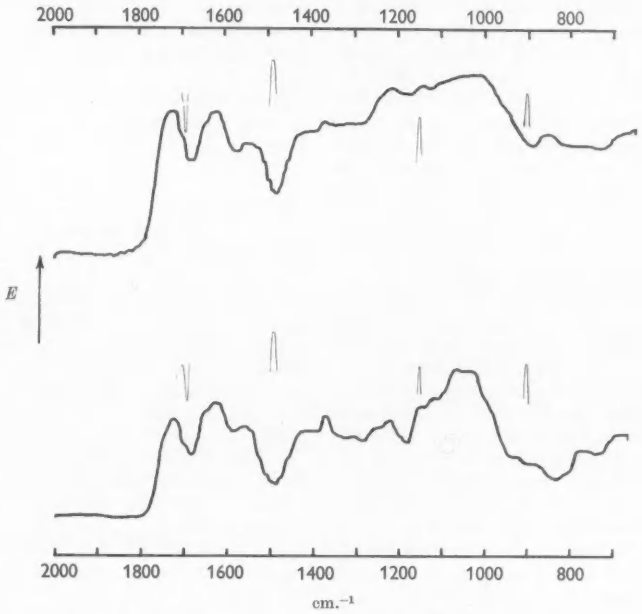


Fig. 3. Infrared spectra. Upper curve, teichuronic acid; lower curve, chondroitin. Each compound was examined in the form of the free acid.

way to distinguish it from other proteins. Also in the wall is a much larger amount of mucopeptide similar to that in wall preparations from other micro-organisms. This mucopeptide contains glutamic acid, alanine, $\alpha\epsilon$-diaminopimelic acid, hexosamine and 3-*O*-carboxyethylglucosamine; it is rendered soluble by the action of egg-white lysozyme. In addition to these components are the polyribitol phosphate–glucose compound, called by Baddiley and his co-workers (Baddiley, 1959; Armstrong *et al.* 1960) teichoic acid, and the mucopolysaccharide described in the present paper as teichuronic acid. Thus in the wall preparations from *B. subtilis* strain 6346 made by a technique similar to that of Cummins & Harris (1956) there are at least four polymeric substances. Nothing, of course, is yet known about the arrangement or importance of these substances for the functioning of the wall surrounding the living organisms. It is known (Salton, 1953; Barkulis & Jones, 1957; Freimer, Krause & McCarty, 1959) that in streptococci, for example, M protein adheres very firmly to the cell-wall material, and it is possible that any substance of the right properties which is destined to be exported from the cell into the external medium may be partially retained by the cell wall. The insoluble protein of the cell wall is presumably present as a structural feature in the organism but the exact status of the apparently water-soluble teichoic acid and teichuronic acid is thus less clear. These may be held in the meshes of the insoluble wall structure by forces other than covalencies and may not play any essential role in maintaining the structure of the wall. On the other hand, even if this is true, the presence of these large electronegatively charged molecules might be expected to have considerable influence on the 'permeability' of the cell wall to other macromolecules such as proteins during the course of their exportation from the cell.

It should also be noted that we have examined only one strain of *B. subtilis* and it seems improbable that one could generalize to other strains of the organism. Nevertheless, sufficient has been done to show that attempts to suggest structures for mucopeptides on the basis of overall analyses of the unfractionated cell-wall preparations are unlikely to be of value because components of different unrelated polymers may all contribute to the final analytical figures. Thus when cell-wall preparations show molar ratios of apparent glucosamine to muramic acid considerably in excess of

1·0, the strong possibility should be considered that polymers such as mucopolysaccharides or teichoic acids as well as mucopeptide might be present; teichoic acids from organisms such as *Staphylococcus aureus* contain *N*-acetylglucosamine in place of glucose (Baddiley, 1959). Likewise molar ratios of alanine to glutamic acid present in cell-wall material should be scrutinized to see that none of the alanine is contributed by teichoic acid rather than by mucopeptide. Cell-wall material may contain up to 60% or more of teichoic acid, which may contribute considerable amounts of D-alanine according to the method of preparation.

The mucopolysaccharide that is described here yields galactosamine and glucuronic acid when it is hydrolysed under the appropriate conditions. The amino sugar is likely to have been present in the original material as the *N*-acetyl compound since *N*-acetylgalactosamine could be recognized as a constituent of partial hydrolysates made by treatment with dilute acid. Together *N*-acetylgalactosamine and glucuronic acid make up about 80–90% of the weight of dried samples. The remaining small amount of the material is presumably ash.

The relationship of this substance to other known mucopolysaccharides is one of great interest because the components, *N*-acetylgalactosamine and glucuronic acid, are the same as those in chondroitin (Davidson & Meyer, 1955). This latter substance was isolated from the cornea of bovine eyes, and can also be produced by removing the sulphate from chondroitin 4- or 6-sulphate (Kantor & Schubert, 1957). The latter substances are prominent components in mammalian connective tissue. Teichuronic acid differs from chondroitin in the following ways: (*a*) direction of optical rotation; (*b*) ease of acid hydrolysis; (*c*) possession of an absorption band at about 850 cm.$^{-1}$; (*d*) resistance to testicular hyaluronidase. On the basis of these differences we would suggest the possibility that whereas the glycosidic bonds in chondroitin are $\beta$, those in teichuronic acid are $\alpha$ in configuration; the evidence for this is, of course, not complete.

The position of at least some of the glucuronosyl-*N*-acetylgalactosamine bonds is likely to be 1–3, as judged by the reaction of partial acid hydrolysates in the Cifonelli & Dorfman (1958) test. Unfortunately the amounts present under all the hydrolytic conditions examined were so small that no isolation could even be attempted. No evidence has been obtained about the position of the *N*-acetylgalactosaminosyl–glucuronic acid bond.

The properties and composition of teichuronic acid clearly distinguish it from the polysaccharide isolated also from *B. subtilis* cells by Sharon (1957). The material isolated by this author gave no reaction for hexuronic acids and contained galactose and the diamino hexose later identified by Sharon & Jeanloz (1958, 1960). It was isolated from whole cells by extracting them with trichloroacetic acid in a manner similar to that used here for extracting teichuronic acid from cell walls.

## SUMMARY

1. Cell-wall preparations made from vegetative cells of *Bacillus subtilis* strain 6346 contain at least four polymers: (*a*) a mucopeptide consisting of glucosamine, 3-carboxyethylglucosamine (muramic acid), glutamic acid, $\alpha\epsilon$-diaminopimelic acid, alanine and probably a trace of glycine; (*b*) an insoluble protein; (*c*) teichoic acid; (*d*) a mucopolysaccharide called here teichuronic acid.

2. Teichuronic acid consists of equimolar amounts of *N*-acetylgalactosamine and glucuronic acid.

3. The glycosidic linkage between at least part of the glucuronic acid and the *N*-acetylgalactosamine in teichuronic acid is likely to be on C-3 of the amino sugar.

4. The properties of teichuronic acid strongly suggest that the glycosidic bonds are $\alpha$ in configuration.

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# The Specificity of Malic Dehydrogenase from Higher Plants

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Evidence has been presented (Davies & Kun, 1957) supporting the view that malic dehydrogenase present in ox-heart mitochondria is an α-hydroxydicarboxylic acid dehydrogenase, catalysing the following reactions between substrate and diphosphopyridine nucleotide ($DPN^+$):

L-Malate + $DPN^+$
⇌ oxaloacetate + reduced DPN (DPNH) + $H^+$ (1)

D(−)- or *meso*-Tartrate + $DPN^+$
⇌ oxaloglycollate + DPNH + $H^+$ (2)

Oxaloglycollate + $DPN^+$
⇌ dioxosuccinate + DPNH + $H^+$ (3)

Tartronate (hydroxymalonate) + $DPN^+$
⇌ mesoxalate (oxomalonate) + DPNH + $H^+$ (4)

α-Hydroxyglutarate + $DPN^+$
⇌ α-oxoglutarate + DPNH + $H^+$ (5)

Reactions (1)–(4) have been demonstrated in higher plants and tentative evidence that separate enzymes catalyse reactions (1), (2) and (4) has been presented (Stafford, 1956, 1957). Whilst admitting that proof of specificity requires the separation of enzyme activities Stafford produced two types of evidence to support the view that malic dehydrogenase and hydroxymalonic dehydrogenase are separate enzymes: (*a*) During the purification of hydroxymalonic dehydrogenase, the relative activities with oxomalonate, dioxosuccinate and oxaloacetate were not constant; (*b*) hydroxymalonate is a competitive inhibitor of malic dehydrogenase.

It is probable that the concentration of oxomalonate and dioxosuccinate used by Stafford was well below saturation and in consequence her assays would be sensitive to errors in concentration of the unstable keto acids. The observation of competitive inhibition, contrary to Stafford's argument, supports the view that a single enzyme is involved and quantitative evidence for this view will be presented.

Delbruck, Schimassek, Bartsch & Bücher (1959) have suggested that animal cells contain two distinct malic dehydrogenases: one located in the

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mitochondria, the other being a soluble or supernatant enzyme. Malic dehydrogenase is similarly distributed in plant cells (Price & Thimann, 1954) and this paper reports a comparison of the enzymes from the two intracellular locations.

## MATERIALS AND METHODS

*Calcium phosphate gel.* This was prepared by the method of Keilin & Hartree (1938).

*Keto acids.* These were obtained from L. Light and Co. Ltd. and on the basis of chromatography of their 2:4-dinitrophenylhydrazones were judged pure.

*Analytical methods.* Spectrophotometric measurements were made with a Unicam SP. 500 instrument, fitted with a constant-temperature compartment. Silica cells of 1 cm. light-path and 3 ml. capacity were used for the routine assay of enzyme activity, and cells of 4 cm. light-path were used for kinetic studies. DPNH was determined by measurement of light-absorption at 340 mμ. Protein was determined by the method of Warburg & Christian (1942).

*Preparation of malic dehydrogenase from mitochondria and 'supernatant'.* Mitochondria were isolated from the epicotyls of peas (*Pisum sativum* var. Alaska) by the method of Millerd & Bonner (1954). The mitochondrial pellet was washed three times before being suspended in potassium phosphate buffer (0·01 M, pH 7·4) and frozen by a solid carbon dioxide–acetone mixture. The extract was thawed under running tap water, and, after freezing and thawing once more, the extract was centrifuged at 30 000*g* for 15 min. The clear supernatant was collected, brought to 50% saturation with ammonium sulphate and after 10 min. the precipitated protein was collected by centrifuging, dissolved in phosphate buffer (0·2 M, pH 7·0) and dialysed for 2 hr. against the same buffer.

The supernatant obtained after removing mitochondria from the homogenate was also brought to 50% saturation and the precipitated protein dissolved and dialysed as above.

*Enzyme assays.* (*a*) Dehydrogenase activity was measured at 30° by following the oxidation of DPNH in silica cells of light-path 1 cm. containing potassium phosphate buffer (pH 7·0, 0·2 M), the disodium salt of ethylenediaminetetraacetic acid (3 mg.), DPNH (0·4 μmole), enzyme (0·2 ml. diluted to give an extinction change of not more than 0·07/min.) and oxaloacetate (0·5 mM), dioxosuccinate (10 mM) or oxomalonate (25 mM), in a total volume of 3 ml. Under these conditions the velocity is proportional to the enzyme concentration. A unit of activity was defined as the amount of enzyme producing an extinction change of 1·0/min. and specific activity is defined as the number of units/mg. of protein.

(*b*) Glutamic–oxaloacetic transaminase was measured at 20° by following the increase in absorption at 280 mμ in a silica cell of 1 cm. light-path containing phosphate buffer (0·2 M, pH 7·0), aspartate (0·05 M), and oxoglutarate (0·025 M), pyridoxal phosphate (0·1 mM) and enzyme in a total volume of 3 ml. Enzyme (0·1–0·5 ml.) was incubated with pyridoxal phosphate and aspartate for 10 min. before adding buffer. The reaction was started by the addition of α-oxoglutarate. Under these conditions, a unit of activity is defined as the amount of enzyme giving an extinction change of 0·01/min.

*Purification of malic dehydrogenase.* During the purification of glutamic–oxaloacetic transaminase from cauliflowers (R. J. Ellis & D. D. Davies, in preparation), it was observed that malic dehydrogenase was closely associated with the transaminase and in particular the two enzymes overlapped during ion-exchange chromatography on a column of *NN*-diethylaminoethylcellulose (DEAE-cellulose) (Fig. 1). After combining the fractions of the main peak of activity, a preparation from cauliflowers gave a specific activity of 100. This observation formed the basis of a method for the purification of malic dehydrogenase from pea epicotyls.

Peas were grown in darkness at 25° for 7 days, and the epicotyls were removed and blended with ice-cold potassium phosphate buffer (pH 7·4, 0·2 M) in a Waring Blendor, to give a 1:1 (w/v) homogenate. The homogenate was strained through a linen towel and after cooling to 3–5° $(NH_4)SO_4$ was added to give 50% saturation. After 10 min., the precipitated protein was collected by centrifuging at 25 000*g* for 5 min., dissolved in potassium phosphate buffer (pH 7·4, 0·2 M) and dialysed for 1 hr. against a flow of phosphate buffer (pH 7·4, 0·05 M). The solution was centrifuged clear and then serially treated with $(NH_4)_2SO_4$ to give fractions precipitated at 0–25, 25–35, 35–45 and 45–55% saturation. The precipitate from each fraction was collected by centrifuging, dissolved in phosphate buffer (pH 7·4, 0·05 M) and assayed for activity. The fraction with the main activity (35–45%) was stored overnight at −15°. After thawing, the active fraction was dialysed for 2 hr. against a flow of 15 l. of a solution of $K_2HPO_4$ (1 mM). Calcium phosphate gel (8·3 mg. dry wt./ml.) was added in successive small portions (0·1 vol.) and after each addition the suspension was kept for 10 min. before the gel was removed by centrifuging and the supernatant was assayed for enzyme activity. Further additions of gel were made until the specific activity of malic dehydrogenase reached a maximum. The clear solution was then poured on a column of DEAE-cellulose, previously equilibrated with a solution of $K_2HPO_4$ (2 mM). The column

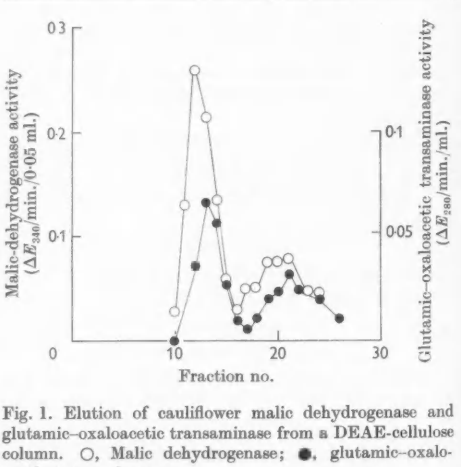



Fig. 1. Elution of cauliflower malic dehydrogenase and glutamic–oxaloacetic transaminase from a DEAE-cellulose column. ○, Malic dehydrogenase; ●, glutamic–oxaloacetic transaminase.

Table 1. *Purification of malic dehydrogenase from pea epicotyls*

Enzyme activity was measured at pH 7·0 by measuring the oxidation of DPNH by oxaloacetate at 340 mμ. A unit of activity is the amount of enzyme producing an extinction change of 1·0/min. Activity ratios for α-oxodicarboxylic acids were determined as described in the text.

| Fraction | Vol. (ml.) | Total activity (units) | Total protein (mg.) | Specific activity (units/mg.) | Recovery (%) | Purification factor | Oxaloacetate / Dioxosuccinate | Oxaloacetate / Oxomalonate |
|---|---|---|---|---|---|---|---|---|
| Extract | 1500 | 75 400 | 16 200 | 4·6 | — | — | 9·2 | 14·2 |
| 1st $(NH_4)_2SO_4$ precipitate | 220 | 22 500 | 2 100 | 10·7 | 30 | 2·3 | 9·9 | 12·1 |
| 2nd $(NH_4)_2SO_4$ precipitate | 50 | 14 200 | 350 | 40·6 | 19 | 9 | 10·2 | 12·0 |
| Gel supernatant | 72 | 12 300 | 180 | 67 | 16 | 15 | 10·2 | 12·7 |
| Combined fractions from DEAE-cellulose column | 60 | 4 100 | 17 | 240 | 6 | 52 | 10·0 | 11·9 |

Table 2. *Activity ratios with α-oxodicarboxylic acids*

| Fraction | Specific activity | Oxaloacetate / Dioxosuccinate | Oxaloacetate / Oxomalonate |
|---|---|---|---|
| Fractions obtained during the purification of glutamate–oxaloacetate transaminase from cauliflowers | | | |
| DEAE-cellulose eluate | 100 | 10·9 | 12·8 |
| DEAE-cellulose eluate | 65 | 10·5 | 12·7 |
| Gel supernatant | 10 | 10·0 | 13·2 |
| Fractions isolated from a homogenate of pea epicotyls | | | |
| Mitochondria | 58 | 10·5 | 13·7 |
| Supernatant | 4 2 | 9·2 | 12·2 |

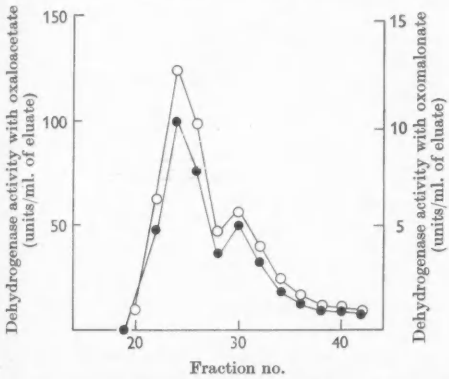



Fig. 2. Chromatography of malic dehydrogenase from pea epicotyls on DEAE-cellulose. Fractions (10 ml.) were collected and assayed for activity with oxaloacetate and oxomalonate. ○, Activity with oxaloacetate; ●, activity with oxomalonate.

was subjected to gradient elution with a mixing volume of 1 l. of $K_2HPO_4$ (2 mM) and a reservoir of phosphate buffer (0·2 M, pH 8·0). The column eluate was collected in 10 ml. fractions and the fractions were assayed for activity. The tubes containing the peak of enzyme activity were combined and stored at −15°; little loss of activity occurred over a period of 9 months. The results of the purification are shown in Table 1.

## RESULTS

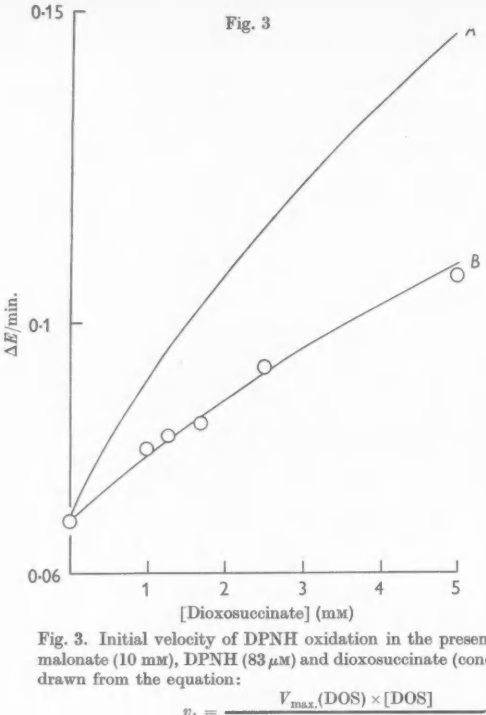
### *Specificity of malic dehydrogenase*

Evidence that a single protein catalyses the reduction of oxaloacetate, dioxosuccinate and oxomalonate was obtained by comparing the activity ratios during purification, by comparing these ratios in preparations derived from mitochondria and supernatant, and by a kinetic study of the interaction of substrates with the enzyme.

*Activity ratios.* Table 1 shows that the activity ratios with different substrates were, within the limits of experimental error, constant throughout the purification. Cauliflower preparations also gave the same activity ratios (Table 2), and where two peaks of malic dehydrogenase appeared after ion-exchange chromatography (Fig. 2) the ratios were also constant.

*Mixed substrates.* Stafford's observation of competitive inhibition is in agreement with the hypothesis of a single enzyme. Thorn (1949) and independently Whittaker & Adams (1949) have shown that the rate of reaction with two substrates is given by the equation:

$$v_t = \frac{V_{max.}(1)\,[S_1]}{[S_1]+K_m(1)\left(1+\frac{[S_2]}{K_m(2)}\right)} + \frac{V_{max.}(2)\,[S_2]}{[S_2]+K_m(2)\left(1+\frac{[S_1]}{K_m(1)}\right)}.$$



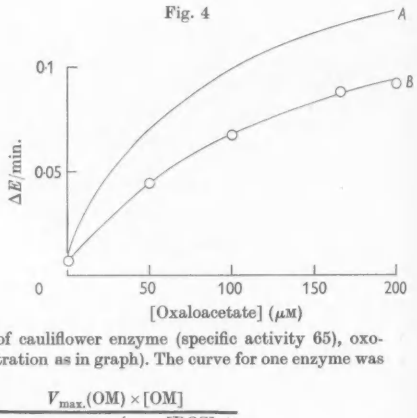




Fig. 3. Initial velocity of DPNH oxidation in the presence of cauliflower enzyme (specific activity 65), oxomalonate (10 mM), DPNH (83 $\mu$M) and dioxosuccinate (concentration as in graph). The curve for one enzyme was drawn from the equation:

$$v_t = \frac{V_{max.}(DOS) \times [DOS]}{[DOS] + K_m(DOS)\left\{1 + \frac{[OM]}{K_m(OM)}\right\}} + \frac{V_{max.}(OM) \times [OM]}{[OM] + K_m(OM)\left\{1 + \frac{[DOS]}{K_m(DOS)}\right\}}$$

$$= \frac{0{\cdot}2 \times [DOS]}{[DOS] + 8 \times 10^{-3}\left(1 + \frac{1}{2{\cdot}5}\right)} + \frac{0{\cdot}208 \times 10^{-2}}{10^{-2} + 2{\cdot}5 \times 10^{-2}\left(1 + \frac{[DOS]}{8 \times 10^{-3}}\right)}.$$

The curve for two enzymes was calculated from the equation:

$$v_t = \frac{V_{max.}(DOS) \times [DOS]}{[DOS] + K_m(DOS)} + \frac{V_{max.}(OM) \times [OM]}{[OM] + K_m(OM)}$$

$$= \frac{0{\cdot}2 \times [DOS]}{[DOS] + 8 \times 10^{-3}} + \frac{0{\cdot}208 \times 10^{-2}}{10^{-2} + 2{\cdot}5 \times 10^{-2}}$$

where DOS is dioxosuccinate and OM is oxomalonate. Experimental values are indicated by ○. *A*, Curve calculated for two enzymes; *B*, curve calculated for one enzyme.

Fig. 4. Initial velocity of DPNH oxidation in the presence of pea enzyme (specific activity 240), DPNH (83 $\mu$M), oxomalonate (0·16 mM), and oxaloacetate (concentration as in graph). The curve for one enzyme was drawn from the equation:

$$v_t = \frac{V_{max.}(OAA) \times [OAA]}{[OAA] + K_m(OAA)\left\{1 + \frac{[OM]}{K_m(OM)}\right\}} + \frac{V_{max.}(OM) \times [OM]}{[OM] + K_m(OM)\left\{1 + \frac{[OAA]}{K_m(OAA)}\right\}}$$

$$= \frac{0{\cdot}166 \times [OAA]}{[OAA] + 8 \times 10^{-5}\left(1 + \frac{1{\cdot}66}{1{\cdot}4}\right)} + \frac{0{\cdot}014 \times 1{\cdot}66 \times 10^{-2}}{1{\cdot}66 + 1{\cdot}4 \times 10^{-2}\left(1 + \frac{[OAA]}{8 \times 10^{-5}}\right)}.$$

The curve for two enzymes was drawn from the equation:

$$v_t = \frac{V_{max.}(OAA) \times [OAA]}{K_m(OAA) + [OAA]} + \frac{V_{max.}(OM) \times [OM]}{K_m(OM) + [OM]}$$

$$= \frac{0{\cdot}166 \times [OAA]}{8 \times 10^{-5} + [OAA]} + \frac{0{\cdot}014 \times 1{\cdot}66 \times 10^{-2}}{14 \times 10^{-2} + 1{\cdot}66 \times 10^{-2}}$$

where OM is oxomalonate and OAA is oxaloacetate. Experimental values are indicated by ○. *A*, Curve calculated for two enzymes; *B*, curve calculated for one enzyme.

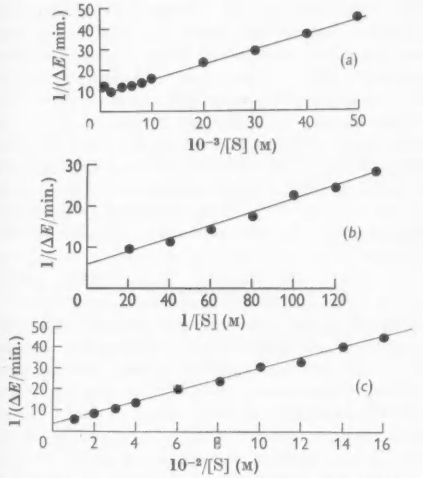



Fig. 5. Relationship between initial velocity of DPNH oxidation and concentration of keto acids. The enzyme was obtained from cauliflower and had a specific activity of 65. The activity was measured at 30° in 4 cm. cells containing DPNH (1 μmole), phosphate buffer (0·2 M, pH 7·0), keto acid as indicated and enzyme in a total volume of 12 ml. (*a*), Oxaloacetate, $K_m$ 90 μM; (*b*), oxomalonate, $K_m$ 25 mM; (*c*), dioxosuccinate, $K_m$ 8 mM.

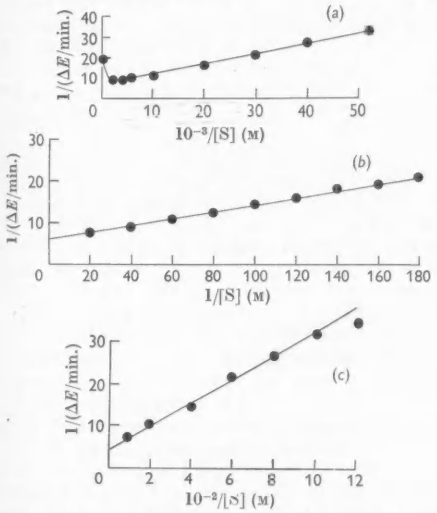



Fig. 6. Relationship between initial velocity of DPNH oxidation and concentration of keto acids. The enzyme was obtained from peas and had a specific activity of 240. The activity was measured as in Fig. 5. (*a*), Oxaloacetate, $K_m$ 90 μM; (*b*), oxomalonate, $K_m$ 25 mM; (*c*), dioxosuccinate, $K_m$ 7 mM.

The rate of reaction with two substrates if separate enzymes are involved is given by:

$$v_i = \frac{V_{max.}(1)\,[S_1]}{[S_1]+K_m(1)} + \frac{V_{max.}(2)\,[S_2]}{[S_2]+K_m(2)},$$

where $V_{max.}$ represents the velocity in the presence of a saturating concentration of substrate ([S]) and $K_m$ the Michaelis constant, i.e. the substrate concentration giving half-maximum velocity. The effect on the rate of oxidation of DPNH, when one substrate is varied and the other is kept constant, has been calculated for a single enzyme and for two enzymes. The rate determinations shown in Figs. 3 and 4 are in good agreement with the hypothesis of a single enzyme.

*Kinetics.* The effect of varying the substrate concentration on the rate of oxidation of DPNH was studied with the three keto acids and with enzyme preparations obtained from cauliflowers and peas. The results in Figs. 5 and 6 show that, within experimental error, the $K_m$ values for the individual substrates are the same for enzymes prepared from cauliflowers and from peas.

The effect of varying the concentration of DPNH on the rate of reduction of oxomalonate and oxaloacetate was determined and the results are illustrated in Fig. 7; the difference between the

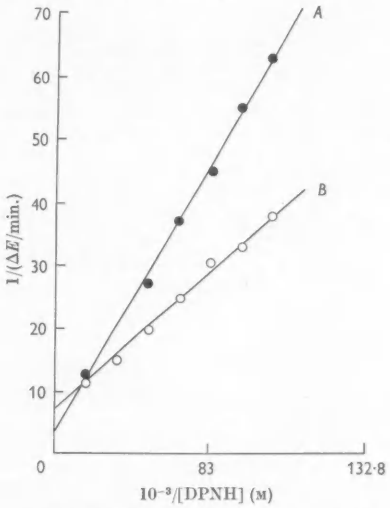



Fig. 7. Relationship between initial velocity of DPNH oxidation and concentration of DPNH, in the presence of constant concentrations of either (*A*) oxaloacetate (50 μM) or (*B*) oxomalonate (16·6 mM) and pea malic dehydrogenase (specific activity 240). The initial velocities were measured in 4 cm. cells. *A*: $K_{DPNH}$ in the presence of oxaloacetate, 120 μM; *B*: $K_{DPNH}$ in the presence of oxomalonate, 35 μM.

values of $K_{DPNH}$ is greater than experimental error. Substrate inhibition was observed with oxaloacetate concentrations in excess of 0·5 mM.

*Different forms of malic dehydrogenase.* Preparations of malic dehydrogenase from mitochondria and supernatant were compared with respect to the effect of oxaloacetate concentration on reaction velocity. The results presented in Fig. 8 show differences between the preparations, but it is difficult to assess their significance. The possibility that malic dehydrogenase from the two cellular locations differs in substrate specificity was investigated by comparing the activity ratios. The results shown in Table 2 do not suggest any significant differences between the two preparations. The purified preparation of malic dehydrogenase was made from a homogenate in which approximately 75% of the activity was derived from the supernatant and 25% from the mitochondria.

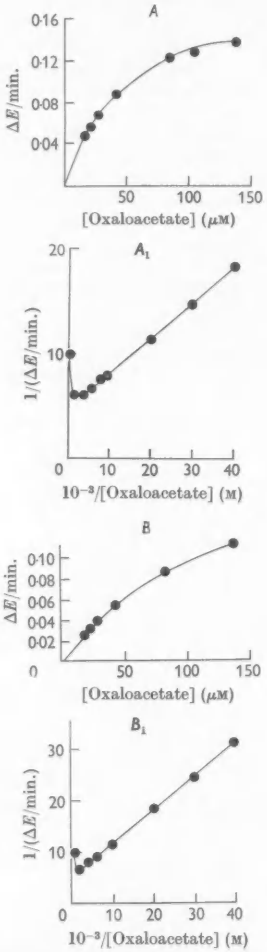



Fig. 8. Relationship between initial velocity of oxaloacetate reduction and concentration of oxaloacetate with (*A*) enzyme obtained from pea supernatant ($K_m$ for oxaloacetate, 74 μM) and (*B*) enzyme obtained from pea mitochondria ($K_m$ for oxaloacetate, 125 μM). The initial velocities were measured as in Fig. 5.

## DISCUSSION

The experimental evidence supports the view that plant malic dehydrogenase should be considered an α-hydroxydicarboxylic acid dehydrogenase. The evidence presented by Stafford (1956) for the existence of a specific hydroxymalonic dehydrogenase may be explained as due to the failure to approach substrate saturation in the development of the enzyme assays and to a mistaken interpretation of competitive inhibition. The finding that the Michaelis constant for DPNH in the presence of oxaloacetate is not the same as in the presence of oxomalonate is not at variance with the hypothesis of a single enzyme. If $K_{DPNH}$ represents a true dissociation constant

$$(K_{DPNH} = [E]\,[DPNH]/[E\text{–}DPNH])$$

then its value should be independent of the second substrate. However, it is probably true that in few cases is the equilibrium assumption of Michaelis & Menten (1913) completely valid and $K_{DPNH}$ is a more complex function involving a velocity constant for the second substrate.

In recent years, attention has been drawn to the apparent heterogeneity of many enzymes and the views of a number of investigators would seem to be embodied in the following quotation: 'The concept of enzyme specificity goes far beyond substrate specificity and is to be extended to cover physiological specificity in the broadest sense' (Hess, 1958). With reference to malic dehydrogenase, electrophoresis of serum has indicated the presence of three peaks with malic-dehydrogenase activity (Vessel & Bearn, 1958). A possible physiological difference between the dehydrogenases present in the mitochondria and in the cytoplasm has been suggested by Delbruck *et al.* (1959), who confirmed the finding of Davies & Kun (1957) that the mitochondrial enzyme is inhibited by concentrations of oxaloacetate greater than 0·1 mM, and observed that the cytoplasmic dehydrogenase is not inhibited by concentrations of oxaloacetate up to 1 mM. The possibility that the kinetic differences between the enzymes is reflected in molecular structure is suggested by the

finding that, on electrophoresis, 90% of the supernatant enzyme migrates to the anode, whereas the anode component of the mitochondrial enzyme represents only 14% of the total (Wieland, Pfleiderer, Haupt & Worner, 1959). Evidence for two components with malic-dehydrogenase activity was obtained during chromatography of preparations from cauliflowers and peas (Figs. 1 and 2), but the separation did not appear to be connected with differences in specificity (Table 2).

It appears to the author that the existence of protein interactions and the likelihood of proteolytic activity modifying the structure of enzymes without destroying catalytic activity make it quite likely that electrophoresis or chromatography will indicate the presence of two peaks of activity in many enzyme preparations. The observation of multiple peaks may be unimportant unless a physiological difference between the molecular species of the enzyme is found. The existence of different enzymes catalysing the same reaction is potentially of great interest. The discovery of two examples in bacteria, the existence of two threonine deaminases, one of which is biosynthetic whereas the other is adaptive and degradative (Umbarger & Brown, 1957), and the demonstration of two distinct systems for the synthesis of acetolactate, only one of which appears to participate in valine biosynthesis (Halpern & Umbarger, 1959), suggest that the pattern of two enzymes catalysing the same reaction may be found in other biological systems.

## SUMMARY

1. A method of purifying malic dehydrogenase from pea epicotyls is described. A 50-fold purification was obtained by ammonium sulphate fractionation, treatment with calcium phosphate gel and chromatography on diethylaminoethylcellulose.

2. Evidence is presented for the view that plant malic dehydrogenase, like the corresponding animal enzyme, is an α-hydroxydicarboxylic acid dehydrogenase.

3. A number of kinetic constants have been evaluated.

4. Slight differences between malic dehydrogenase prepared from mitochondria and from the supernatant remaining after removing mitochondria from a homogenate were noted. Two peaks with malic-dehydrogenase activity were observed during chromatography but all preparations appeared to have the same substrate specificity.

I wish to thank Professor T. A. Bennet-Clark, F.R.S., for advice, Mr R. J. Ellis for carrying out the assays of glutamic–oxaloacetic transaminase, Miss J. Bloom for technical assistance, and The Royal Society and the Agricultural Research Council for grants towards the cost of apparatus.

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# The Determination of Magnesium in Biological Materials by Atomic Absorption Spectrophotometry

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The wide variety of methods which have been employed for the determination of magnesium in biological materials serves to illustrate the fact that no method is completely satisfactory. The majority of methods are chemical and the earlier procedures require an initial separation of magnesium, either as the insoluble ammonium phosphate salt (Briggs, 1922; Denis, 1922), or as the complex with 8-hydroxyquinoline (Greenberg & Mackey, 1932). Direct combination with the dye

* Medical Research Council External Staff.

Titan yellow has been widely used (Gillam, 1941; Garner, 1946; Orange & Rhein, 1951), whereas more recently methods utilizing other dyestuffs and titration with ethylenediaminetetra-acetic acid have been developed (Smith, 1955; Wilkinson, 1957). Unfortunately many of these methods are inaccurate or of very limited application, owing to interference by other constituents of biological materials.

It is desirable that any method should be applicable to the analysis of ashed specimens of faeces and food or other cellular material in addition to biological fluids such as serum and urine. Modifications of existing ammonium phosphate-separation methods have been shown to yield satisfactory results with all types of specimen (Heaton, 1960), but as the analysis requires 2 days a more rapid method is necessary for many purposes.

During the past few years flame photometry has been applied to the problem (Vallee & Margoshes, 1956; MacIntyre & Davidsson, 1958), but the method is of low sensitivity, owing to the weak emission of the magnesium ion compared with the flame background, unless either very high flame temperatures or integrating systems are employed (Exley & Sproat, 1958). Greater sensitivity may be obtained by utilizing the principle of atomic absorption spectroscopy suggested by Walsh (1955). This principle has been used by Allan (1958) and David (1958) to determine several metals in biological materials after preliminary ashing, and by Willis (1960*a*, *b*) to estimate calcium and magnesium in serum after simple dilution.

The estimation of magnesium by atomic absorption spectroscopy involves passing light containing a high proportion of magnesium resonance radiation through a flame. Into the flame are aspirated solutions whose magnesium content is to be determined, and the light absorbed at 285.213 mμ is compared with the absorption of a standard magnesium solution treated similarly. An instrument suitable for atomic absorption spectrophotometry has been constructed in this laboratory and the method examined for the analysis of both biological fluids and solutions of the ash obtained from cellular materials.

The classical magnesium ammonium phosphate separation method has been critically examined and its accuracy determined. By comparing the results of this method with those obtained from the same specimens analysed by atomic absorption spectrophotometry, the absolute accuracy of the latter method has been estimated.

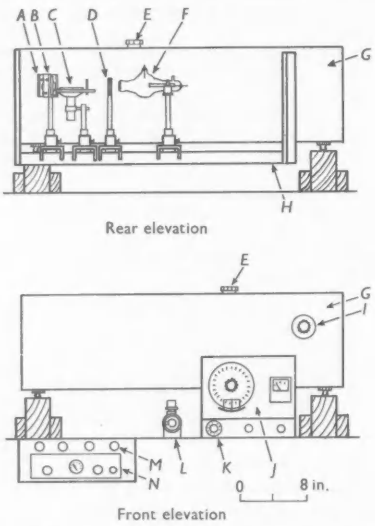


Fig. 1. Arrangement of atomic absorption spectrophotometer. *A*, Entrance slit of monochromator; *B*, mirror; *C*, burner; *D*, condenser lens; *E*, wavelength control; *F*, magnesium lamp; *G*, monochromator and detection system; *H*, optical bench; *I*, slit-width control; *J*, extinction scale and null-point meter; *K*, needle valve; *L*, atomizer and spray chamber; *M*, sensitivity controls; *N*, lamp-current controls.

## EXPERIMENTAL

### *Description of the atomic absorption spectrophotometer*

The arrangement of the principal components (the magnesium lamp, atomizer, spray chamber, burner, monochromator and detection system) is shown in Fig. 1.

*Magnesium lamp.* The lamp, generating light of the magnesium resonance frequency, is a quartz-windowed,

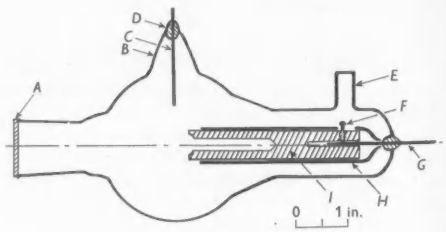


Fig. 2. Magnesium lamp. *A*, Quartz window sealed to glass envelope with black wax; *B*, glass envelope; *C*, tungsten anode; *D*, C79 glass-to-metal seal; *E*, sealed-off connexion to vacuum system; *F*, screw locking cathode in position; *G*, tungsten-rod support for cathode; *H*, hard-glass sleeve; *I*, hollow magnesium cathode.

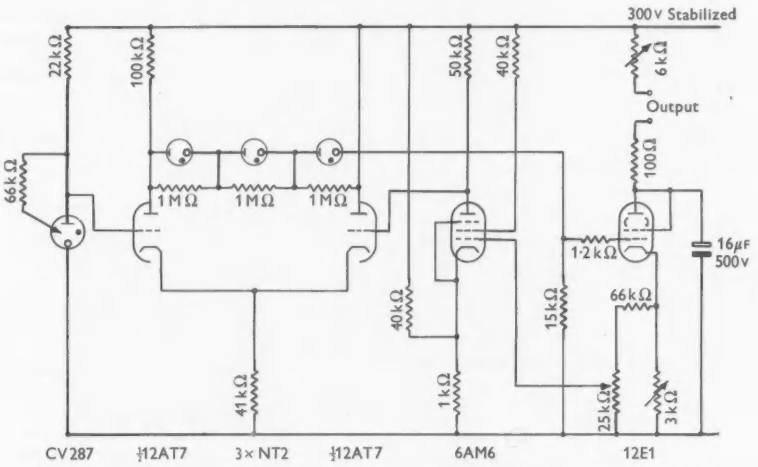


Fig. 3. Circuit diagram of current-regulated power supply for magnesium lamp.

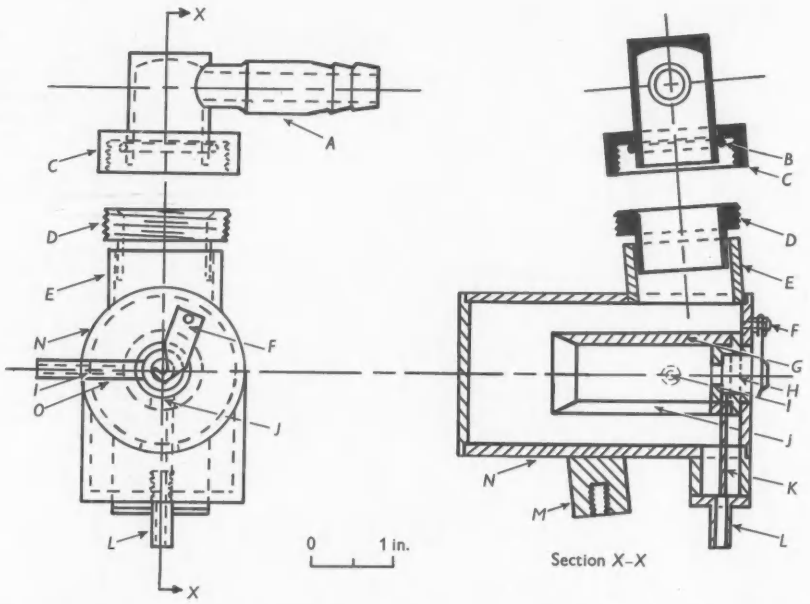


Fig. 4. Spray chamber. *A*, Connexion to burner; *B*, O-ring seal; *C*, knurled locking ring; *D*, threaded brass flange; *E*, Perspex tube at angle of 5° to main body; *F*, clip to secure EEL atomizer; *G*, inner Perspex cylinder; *H*, recess for atomizer; *I*, acetylene inlet; *J*, slit in inner cylinder for drainage; *K*, rod guiding drops to outlet; *L*, drain; *M*, block fastening chamber to bench; *N*, outer Perspex cylinder; *O*, groove to accommodate side arm of atomizer.

hollow magnesium-cathode discharge tube, filled with argon at a pressure of 1–2 mm. Hg (Fig. 2). Power is supplied to the lamp by a current stabilizer (Fig. 3), which is fed from a Solartron constant-voltage supply, type AS 517 (Solartron Electronic Group Ltd., Thames Ditton, Surrey).

*Gas supply.* Air and acetylene are supplied from cylinders fitted with two stage regulators (types BOR 12 and BAR 9 respectively, British Oxygen Gases Ltd., Wembley), fine control of the acetylene flow rate being provided by a needle valve situated on the instrument.

*Atomizer.* The atomizer is of the type used in the EEL flame photometer (Evans Electroselenium Ltd., Harlow, Essex).

*Spray chamber.* This is constructed of Perspex (Fig. 4), the drain running into a sealed bottle to help maintain a steady gas pressure in the chamber.

*Burner.* The burner, constructed of brass, is water-cooled to eliminate instability due to temperature variation (Fig. 5). A diaphragm is inserted in the light-path to restrict absorption measurements to the optimum part of the flame.

*Monochromator and detection system.* This consists of the monochromator and detection system of the CF4 spectrophotometer (Optica U.K. Ltd., Gateshead-on-Tyne), the lamp housing being replaced by a parallel-bar optical bench. On the bench are mounted the magnesium lamp, a condenser lens, the burner and a mirror which reflects the light into the entrance slit of the monochromator.

### *Preparation of the atomic absorption spectrophotometer*

The positions of the lamp, lens and mirror are adjusted to pass maximum light into the monochromator (maximum transmission on the extinction scale). The wavelength control is set at 285 m$\mu$ and then finely adjusted to obtain maximum transmission at the magnesium resonance line 285.213 m$\mu$. The slit width is set at 0·2 mm. The position of the burner is adjusted until the gas ports are aligned with the optical axis and 12 mm. below this axis. The instrument is checked to ensure that addition of sodium (17 mg./100 ml.) to a standard magnesium solution (0·05 mg./100 ml.) produces no change in extinction. If interference is observed, the height of the burner is adjusted relative to the optical axis until this effect disappears. The instrument is now correctly assembled, and further adjustment is necessary only after moving any part of the equipment.

The atomizer and gas ports in the burner should be cleaned after approximately 100 estimations, and the drain bottle from the spray chamber must be emptied periodically. No other routine maintenance is required.

### *Operation of the atomic absorption spectrophotometer*

The magnesium lamp is allowed to stabilize for approximately 1 hr. before the instrument is used. Cooling water is then passed through the burner, the pressures of air and acetylene are adjusted to 13 and 5 lb./in.$^2$ respectively, and, with the needle valve fully open, the gas is ignited. The acetylene flow is reduced by means of the needle valve until the white tips just disappear from the cones of unburnt gas in the flame. Water is aspirated into the flame and the sensitivity control is adjusted to give an extinction approaching zero. No attempt is made to adjust exactly to zero as it is impossible to stabilize completely the light-output from the lamp.

Estimations may conveniently be carried out in groups containing up to eight unknown solutions. A standard solution containing 0·050 mg. of magnesium in 100 ml. of 0·1N-hydrochloric acid is measured immediately before and after each group of unknown solutions. The small effect of lamp instability is minimized by spraying water both before and after each magnesium-containing solution, the mean value of these two readings being subtracted from the extinction of the sample. The extinction of every solution is measured twice and, after correction for the distilled

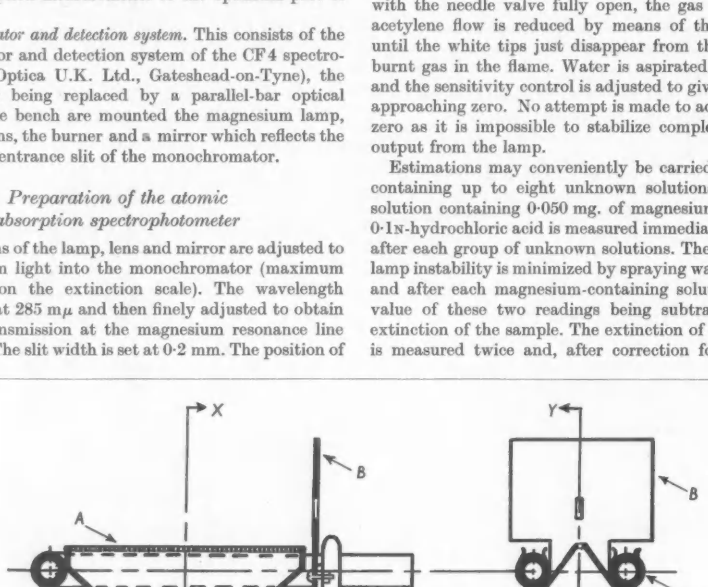


Fig. 5. Burner. *A*, Gas ports (30 × 1 mm. diameter); *B*, metal diaphragm; *C*, water-cooling pipes soldered to body of burner; *D*, Terry clips attaching diaphragm to cooling pipes; *E*, burner body fabricated from 16 s.w.g. sheet brass; *F*, connexion to spray chamber.

water blank, the magnesium content of each unknown is calculated from the ratio of the mean value for its extinction to the corresponding value for the standard solutions.

### *Preliminary treatment of samples*

Specimens are diluted to produce a solution for measurement in the instrument which contains 0·02–0·08 mg. of magnesium/100 ml. Hydrochloric acid (2N) is added to each solution to produce a final concentration of 0·1N.

*Plasma or serum.* Direct, 50-fold dilution is satisfactory.

*Urine.* Dilution 100 times is usually satisfactory.

*Blood cells.* Packed cells are laked with a considerable excess of water before acidification; 100-fold dilution is satisfactory.

*Food and faecal materials.* Solid materials are homogenized with water and diluted to a known volume. Suitable portions are transferred to silica crucibles, dried on a sand bath and ashed overnight in a muffle furnace at 500°. The ash is dissolved in 2N-hydrochloric acid and diluted as described above.

### *Reagents*

*Chemicals.* All materials used were of AnalaR quality with the exception of dipotassium hydrogen phosphate and ethylenediaminetetra-acetic acid (disodium salt), which were of laboratory-reagent grade. The standard calcium solution was prepared from dry calcium carbonate dissolved in the minimum quantity of hydrochloric acid. Glass-distilled water was used for the preparation and dilution of all solutions.

*Standard magnesium solution.* A stock solution of $MgSO_4,7H_2O$ containing 10 mg. of magnesium/100 ml. is employed. Working strength standards of 0·050 mg. of magnesium/100 ml. are prepared by dilution, and acidified with hydrochloric acid to 0·1N in the same manner as the solutions to be analysed. The working standards are stable for 1 week provided they are stored in flasks aged by equilibration with similar solutions.

### *Chemical estimation of magnesium*

Magnesium was determined by the ammonium phosphate separation method of Simonsen, Westover & Wertman (1947) for serum magnesium, modified and extended to the analysis of other materials as described by Heaton (1960).

## RESULTS AND DISCUSSION

### *Development of the method*

*Treatment of solutions.* Magnesium sulphate and magnesium acetate, the two non-deliquescent magnesium salts readily available in a high state of purity, were examined for use as standards. A neutral aqueous solution of magnesium sulphate produced an absorption appreciably greater than that of an equimolar solution of magnesium acetate (Table 1). When these solutions were treated with acetic acid or sulphuric acid in quantities sufficient to equate their anionic composition, an increased extinction was observed in both cases, and the two solutions gave identical results. Similar agreement was obtained when solutions of both salts were acidified with an excess of hydrochloric acid.

Acidification with hydrochloric acid to a concentration of 0·1N was adopted as a routine procedure with both standard and unknown solutions. Under these conditions, a standard solution containing 0·050 mg. of magnesium/100 ml. produced an extinction of approximately 0·10, and the calibration curve was linear up to a magnesium concentration of 0·10 mg./100 ml.

*Selection of correct region in the flame.* The optimum part of the flame for use in atomic absorption measurements was immediately above the cones of unburnt gas. If the burner was too high relative to the optical axis, addition of other cations to a standard magnesium solution caused an apparent enhancement of the magnesium concentration (Table 2).

### *Evaluation of the proposed method*

The evaluation of the atomic absorption method reported in this section was carried out with the height of the burner correctly adjusted, and all solutions were acidified with hydrochloric acid as described in the Experimental section.

*Interference effects.* (*a*) Inorganic ions. The major inorganic constituents of biological materials were tested for interference at the concentrations at which they normally occur, and at twice these levels. Solutions of suitable salts were added to solutions of known magnesium content, and the apparent magnesium concentration was deter-

Table 1. *Influence of anions on the absorption of standard magnesium solutions*

| Solvent | Extinction for 0·050 mg. of Mg/100 ml. | |
|---|---|---|
| | Magnesium sulphate | Magnesium acetate |
| Distilled water | 0·1108 | 0·1058 |
| Acetic acid, 50 $\mu$N | 0·1149 | — |
| Sulphuric acid, 50 $\mu$N | — | 0·1149 |
| Hydrochloric acid, 0·1 N | 0·1125 | 0·1121 |

Table 2. *Effect of burner height on interference produced by other cations*

The magnesium concentration was 0·050 mg./100 ml.

| Distance of burner below optical axis (mm.) | Percentage interference | | |
|---|---|---|---|
| | Sodium (17·0 mg./100 ml.) | Potassium (4·0 mg./100 ml.) | Calcium (0·50 mg./100 ml.) |
| 4 | +4·4 | +3·7 | +1·3 |
| 6 | −0·2 | +3·7 | +0·7 |
| 8 | +0·8 | +2·2 | +1·3 |
| 10 | +1·2 | +2·4 | −0·4 |
| 12 | 0·0 | +0·9 | +0·1 |
| 14 | +0·4 | +0·8 | +1·8 |

mined, making due allowance for the small blank readings of the added salts. The interference observed with different salts was random in nature (Table 3), varying from +1·0 to −1·6 % of the magnesium concentration, and was equal to the experimental error of the measurement.

(*b*) Organic substances. The organic constituents of plasma, blood cells and urine were examined for interference by comparing the magnesium content, measured after simple dilution, with the values obtained after preliminary ashing of the same specimens. Since interference by inorganic ions is negligible the results obtained with ashed material were employed as a standard of reference. Organic material enhanced the apparent magnesium content of all types of specimen (Table 4). With urine and blood cells the enhancement of 0·4 % was insignificant, but with plasma the effect was rather greater (2·3 %).

For the determination of serum magnesium Willis (1960*b*) advocated dilution with solutions of either the disodium salt of ethylenediaminetetra-acetic acid or of strontium chloride to liberate the protein-bound magnesium, which normally constitutes approximately 35 % of the total present in serum (Copeland & Sunderman, 1952; Prasad, Flink, Zinneman & McCollister, 1958). Acidification with hydrochloric acid, however, produced results similar to those obtained by these procedures (Table 5), and simple aqueous dilution yielded

Table 3. *Interference effects of inorganic ions*

The magnesium concentration was 0·0500 mg./100 ml.

| Cation | Concn. (mg./100 ml.) | Anion | Concn. (mg./100 ml.) | Apparent Mg concn. (mg./100 ml.) | Percentage interference |
|---|---|---|---|---|---|
| $Na^+$ | 8·5 | $Cl^-$ | 13·1 | 0·0498 | −0·4 |
| | 17·0 | | 26·2 | 0·0499 | −0·2 |
| $K^+$ | 1·96 | $PO_4^{3-}$ | 2·39 | 0·0505 | +1·0 |
| | 4·01 | | 4·88 | 0·0502 | +0·4 |
| $Ca^{2+}$ | 0·25 | $Cl^-$ | 0·44 | 0·0504 | +0·8 |
| | 0·50 | | 0·88 | 0·0499 | −0·2 |
| $NH_4^+$ | 0·76 | $SO_4^{2-}$ | 2·02 | 0·0492 | −1·6 |
| | 1·52 | | 4·04 | 0·0493 | −1·4 |
| $Fe^{2+}$ | 1·0 | $SO_4^{2-}$ | 1·71 | 0·0502 | +0·4 |
| | 2·0 | | 3·42 | 0·0498 | −0·4 |
| $Fe^{3+}$ | 1·0 | $SO_4^{2-}$ | 1·71 | 0·0502 | +0·4 |
| | 2·0 | | 3·42 | 0·0502 | +0·4 |

Table 4. *Comparison of magnesium analyses carried out by (a) direct dilution and (b) preliminary ashing of the same specimens*

| | Magnesium concn. (mg./100 ml.) | | | | | |
|---|---|---|---|---|---|---|
| | Plasma | | Urine | | Blood cells | |
| | (*a*) | (*b*) | (*a*) | (*b*) | (*a*) | (*b*) |
| | 1·68 | 1·63 | 3·11 | 3·18 | 4·81 | 4·95 |
| | 1·80 | 1·73 | 2·79 | 2·86 | 6·46 | 6·27 |
| | 1·71 | 1·71 | 3·40 | 3·40 | 4·48 | 4·35 |
| | 1·97 | 1·94 | 9·85 | 9·70 | 5·67 | 5·78 |
| | 1·68 | 1·61 | 7·47 | 7·47 | 6·07 | 6·12 |
| | 2·00 | 2·01 | 5·51 | 5·41 | 5·52 | 5·43 |
| Mean | 1·81 | 1·77 | 5·36 | 5·34 | 5·50 | 5·48 |

Table 5. *Effect of various diluents on analysis of plasma*

| Plasma diluent | Extinction: Mg standard (0·050 mg./100 ml.) | Extinction: Plasma (diluted 50 times) | Plasma Mg concn. (mg./100 ml.) |
|---|---|---|---|
| Distilled water | 0·1161 | 0·0920 | 1·98 |
| Hydrochloric acid, 0·1 N | 0·1185 | 0·0910 | 1·92 |
| Ethylenediaminetetra-acetic acid, disodium salt, 10·0 g./l. | 0·1156 | 0·0900 | 1·94 |
| Strontium chloride, 7·6 g./l. | 0·1127 | 0·0872 | 1·93 |

results 2·5% higher. It appears therefore that displacement of the protein-bound magnesium either occurs during aqueous dilution or is not necessary for the atomic absorption method, and the substances used by Willis, in relatively large concentrations, may be acting as spectroscopic buffers in a manner similar to hydrochloric acid.

*Reproducibility and speed.* The average difference between duplicate determinations of the extinction on 40 different solutions was 1·0%. Duplicate estimations of the magnesium content of 20 different specimens varied by an average of 1·5%. This larger error includes both the instrumental error and the volumetric errors involved in the preparation of solutions for assay. After preparation of the solution a single extinction can be measured in approximately 1 min., whereas duplicate measurements of extinctions on a group of ten solutions require 20–30 min.

Table 6. *Comparison of the atomic absorption and magnesium ammonium phosphate methods*

| | Plasma Mg (mg./100 ml.) | | Urine Mg (mg./100 ml.) | |
|---|---|---|---|---|
| | Atomic absorption | Phosphate separation | Atomic absorption | Phosphate separation |
| | 1·87 | 1·78 | 5·44 | 5·33 |
| | 1·92 | 1·99 | 3·11 | 3·07 |
| | 1·81 | 1·75 | 5·78 | 5·53 |
| | 1·64 | 1·68 | 3·39 | 3·27 |
| | 1·74 | 1·71 | 2·79 | 2·83 |
| | 1·61 | 1·68 | 5·32 | 5·14 |
| | 1·89 | 1·81 | 3·09 | 2·99 |
| | 1·68 | 1·69 | 10·50 | 10·80 |
| | 1·83 | 1·80 | 7·44 | 7·29 |
| | 1·66 | 1·59 | 1·86 | 1·69 |
| | 2·08 | 2·00 | 6·05 | 5·77 |
| | 1·97 | 2·02 | 5·11 | 5·18 |
| | 2·24 | 2·09 | 2·67 | 2·40 |
| | 1·96 | 1·95 | 5·40 | 5·58 |
| | 1·71 | 1·67 | 2·67 | 2·65 |
| | 1·80 | 1·73 | 4·86 | 4·88 |
| | 1·68 | 1·63 | 3·40 | 3·40 |
| | 1·96 | 2·00 | 9·56 | 9·66 |
| Mean | 1·84 | 1·81 | 4·91 | 4·86 |
| | Mean difference 0·03 (S.E. 0·01) | | Mean difference 0·05 (S.E. 0·04) | |
| | Food or faecal ash Mg (mg./100 ml.) | | Blood cell Mg (mg./100 ml.) | |
| | Atomic absorption | Phosphate separation | Atomic absorption | Phosphate separation |
| | 6·90 | 7·10 | 5·52 | 5·28 |
| | 4·81 | 4·80 | 5·67 | 5·77 |
| | 5·99 | 6·19 | 6·46 | 6·15 |
| | 4·54 | 4·29 | 4·48 | 4·37 |
| | 5·41 | 5·64 | 4·62 | 4·82 |
| | 4·67 | 4·64 | 5·39 | 5·53 |
| Mean | 5·39 | 5·44 | 5·36 | 5·32 |
| | Mean difference 0·05 (S.E. 0·07) | | Mean difference 0·04 (S.E. 0·08) | |

*Comparison of the atomic absorption and magnesium ammonium phosphate methods.* A micro-modification of the classical ammonium phosphate method has been used as a routine in this laboratory for the determination of magnesium. The principle of this method is the precipitation of magnesium as the ammonium phosphate salt, the magnesium present in the precipitate being calculated from the phosphate content as determined colorimetrically. Calcium is previously removed by precipitation with ammonium oxalate.

Simultaneous analyses were made on 120 different specimens by the proposed method and the ammonium phosphate separation procedure. Typical results are shown in Table 6. The atomic absorption method produced slightly higher results than the chemical method except with inorganic solutions of food and faecal ash, where the average value for the ammonium phosphate method exceeded that for the atomic absorption procedure by 0·9%. With blood cells, urine and plasma the mean values obtained by the proposed method were respectively 0·8, 1·0 and 1·7% higher than the corresponding results from the chemical method.

### *Accuracy of the magnesium ammonium phosphate method*

The systematic errors inherent in this method can be divided into two groups: those which decrease the magnesium content of the final precipitate, and compensatory errors leading to the presence of an enhanced amount of phosphate in the precipitate. The greater sensitivity of the atomic absorption method was used to determine the magnitude of these errors. The magnesium loss was ascertained by determining the total magnesium present in the calcium oxalate precipitate, the supernatant fluid from the magnesium ammonium phosphate precipitate, and the liquid used to wash this precipitate free from excess of phosphate. The magnesium equivalent of the phosphate gain was determined by comparing the magnesium content of the magnesium ammonium phosphate precipitate, as measured directly by atomic absorption spectrophotometry, with the corresponding value obtained by calculation from the phosphate content of this precipitate measured colorimetrically. Suitable blank solutions were measured, and allowance was made for trace contamination introduced by the reagents employed in the chemical procedure. Most of the measurements were carried out on the analyses of serum and urine reported in Table 6.

A total magnesium loss of 2·2–2·5% was observed with both types of material, and this was usually balanced by the phosphate gain, giving results within ± 1% of the true value (Table 7). As

Table 7. *Systematic errors in the magnesium ammonium phosphate method*

Each value is the mean of 20 estimations.

| Material | Magnesium loss (%) | Phosphate gain (%) | Net error (%) |
|---|---|---|---|
| Serum or plasma | 2·5 (S.E. 0·5) | 1·7 (S.E. 0·4) | −0·8 |
| Urine | 2·2 (S.E. 0·4) | 2·3 (S.E. 0·6) | +0·1 |

would be expected, however, the phosphate gain was proportional to the magnesium concentration, which is normally greater in urine than in serum. For serum this gain was slightly less than the magnesium loss and so caused underestimation of the magnesium content by 0·8 %, but with urine the average phosphate gain exceeded the magnesium loss very slightly, producing results 0·1 % high. As the procedures for the analysis of blood cells and solutions of ashed material are identical with the methods for serum and urine respectively, the errors inherent in the latter analyses may be considered typical of those occurring during analysis of the former materials.

Occasionally larger and non-reproducible errors were encountered, owing to disturbance of the magnesium ammonium phosphate precipitate during manipulation. Estimations where such errors occurred were excluded in the compilation of Tables 6 and 7.

### *Absolute accuracy of the atomic absorption method*

Direct comparison with the ammonium phosphate procedure showed that the atomic absorption method produces results 0·8, 1·0 and 1·7 % higher than the chemical method with blood cells, urine and plasma respectively, the mean value for ashed material being 0·9 % below that given by the chemical procedure. If allowance is made for the errors in the chemical method, the results obtained by atomic absorption spectrophotometry for plasma, urine and food or faecal ashes differ respectively by +0·9, +1·1 and −0·8 % from the correct values. These results are not in exact agreement with the probable errors indicated by interference studies, the discrepancy varying from 0·4 % with blood cells to 1·4 % with plasma. No attempt was made to resolve these differences by studying larger series of analyses, as they are sufficiently small to be without practical significance, and do not affect the conclusion that the atomic absorption method is accurate with all types of material to within 2 % of the absolute value.

## SUMMARY

1. An instrument suitable for the determination of magnesium by atomic absorption spectrophotometry is described.

2. Analyses may be carried out on serum, urine, blood cells and solutions of the ash from food or faecal materials after simple dilution and acidification with hydrochloric acid.

3. No significant effect is produced by other inorganic ions. The organic constituents of samples cause slight overestimation of the magnesium concentration.

4. The magnesium ammonium phosphate method is examined and its accuracy determined.

5. Comparison between analyses carried out by the ammonium phosphate method and the atomic absorption procedure shows the latter to be accurate to within 2 % of the absolute value.

We wish to thank Mr D. J. Ellis, Mr S. Taylor and Mr A. W. Chivers for their invaluable help with the construction and evaluation of the instrument.

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# Studies in Immunochemistry

## 19. FURTHER OBSERVATIONS ON THE PREPARATION AND PROPERTIES OF HUMAN BLOOD-GROUP-SPECIFIC MUCOPOLYSACCHARIDES*

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The human blood-group-specific mucopolysaccharides are usually obtained by extracting freeze-dried secretions, such as saliva, gastric juice, ovarian-cyst fluids, meconium or tissue digests with liquid 90 or 95% (w/v) phenol. The active substances are largely insoluble in this solvent. If soluble, however, as they frequently are after liberation from tissues by digestion with pepsin at pH 1–2, they can be readily recovered by the addition of low concentrations of ethanol. Mucopolysaccharides isolated in this way are free from gross contamination with unspecific protein and fatty substances which occur in the native secretions and tissue extracts, and after further purification by fractional precipitation from aqueous solution with suitable organic solvents are the materials which have been most frequently used for chemical and serological studies (cf. Kabat, 1956; Morgan, 1959*a*, 1960).

Until recently these materials were believed to be substantially homogeneous, but a more thorough examination has now shown that in most instances they can be separated into at least two kinds of mucopolysaccharide substance, which, although closely similar in many respects, differ from each other in certain important properties. The method used to bring about the separation depends on the solubility differences of the materials in saturated ammonium sulphate at 60°.

The behaviour of these substances in saturated ammonium sulphate at 60° has been used to develop a simple procedure which allows the specific-blood-group mucopolysaccharides to be isolated from native cyst fluids without the use of phenol and thus enables them to be obtained by a method which is different from that usually employed. Details of this work are given in the present paper.

## EXPERIMENTAL

Cyst fluids obtained from pseudomucinous ovarian cystadenomata, were kept at 1–3° after the addition of toluene or were dried from the frozen state as soon as possible after removal from the patient. The materials were obtained from 'secretor' persons belonging to each of the groups, $A_1$, B, O, $A_1B$ and $A_2B$ and from 'non-secretor' persons. The fluids from this latter class of individual were the source of $Le^a$-specific substance which was free from A, B or H serological character (Grubb & Morgan, 1949; Annison & Morgan, 1952*a*).

*Chemical analyses.* All preparations were thoroughly dialysed against distilled water at 1–3° and dried from the frozen state. The materials were further dried for 30 min. over $P_2O_5$ at 78° and 1–2 mm. Hg pressure before analysis. The methods used for the determination of N, fucose, hexosamine and reducing sugars after acid hydrolysis are as given by Pusztai & Morgan (1961). Quantitative amino acid analyses were made by the method of Moore & Stein (1954) on materials which had been refluxed with constant-boiling HCl on an oil bath for 24 hr. at 135–140°.

*Physical examination.* Ultracentrifugal and electrophoretic examinations were made as described by Kekwick (1950). The relative viscosity was determined at 20° at a concentration of 1% (w/v) in water in an Ostwald-type viscosimeter. The intrinsic viscosities were determined from measurements made at 20° in phosphate buffer (*I* 0·2) containing 0·15 M-NaCl, pH 8.

*Serological examination.* The materials were examined for their capacity to inhibit specific haemagglutination as described by Morgan & King (1943). Naturally occurring anti-A ($\alpha$) and anti-B ($\beta$) agglutinins in normal human sera were used for measuring A and B activity. Immune rabbit anti-A serum and A-specific plant-seed agglutinins (Morgan & Watkins, 1953) were also used as anti-A reagents in the inhibition tests. The H activity (Morgan & Watkins, 1948; Annison & Morgan, 1950; Watkins & Morgan, 1955) was measured by titration with rabbit immune anti-H sera prepared from H substance as described by Annison & Morgan (1952*b*). In certain instances a human anti-H agglutinin (Watkins & Morgan, 1954), an eel anti-H serum (Jonsson, 1944; Watkins & Morgan, 1952) or an H-specific plant-seed agglutinin (Morgan & Watkins, 1953) were also used. The $Le^a$ activity of the materials was measured as described by Grubb & Morgan (1949) and Annison & Morgan (1952*a*) with a human anti-$Le^a$ serum.

The A activity of certain of the A-specific substances was also determined according to the precipitin method of Heidelberger & MacPherson (1943), as developed by Kabat & Bezer (1945).

Group A preparations were examined for their capacity to inhibit the haemolysis of sheep erythrocytes by rabbit anti-A cell haemolytic serum and complement (Morgan & King, 1943).

*Blood-group-specific mucopolysaccharides.* The materials were prepared according to the method given by Gibbons, Morgan & Gibbons (1955), in which dried cyst fluid was extracted with 90 or 95% (w/w) phenol and the phenol-

* Part 18: Pusztai & Morgan (1961).

insoluble residue freed from phenol by thorough dialysis against water. The thick viscous solution obtained was centrifuged to remove any water-insoluble gel and to the clear supernatant fluid was added 2% (w/v) potassium acetate and increasing amounts of ethanol. In most instances, the major part of the material contained in the extract was precipitated between 45 and 55% (w/v) ethanol concentration; the material was immediately dissolved in water to give a 2% (w/v) solution, reprecipitated within the same concentrations of ethanol, and the material obtained thoroughly dialysed against distilled water at 1–3° and dried. Preparations of group-specific mucopolysaccharides made in this way were used for the fractionation experiments described below. Unless stated otherwise, all materials used were insoluble in 90% phenol and soluble in water. The analytical figures for some of the materials studied are given in Table 1.

A typical example of the procedure used to separate these mucopolysaccharide preparations into two closely similar specific substances was as follows. An aqueous 1% (w/v) solution of the purified mucopolysaccharide (3 g.) obtained from cyst fluid no. 332 (group A) was saturated with $(NH_4)_2SO_4$, heated with a slight excess of $(NH_4)_2SO_4$ crystals to about 60° and held at this temperature for about 5 min. A flocculent precipitate formed and was collected after cooling the solution by skimming from the surface, with or without previous centrifuging. Any part of the precipitate detached during this operation was recovered by filtration. Reheating the supernatant solution to 70–80° in the presence of a slight excess of solid $(NH_4)_2SO_4$ gave no further precipitate. The precipitate was thoroughly washed with saturated $(NH_4)_2SO_4$, dissolved in water and dialysed to remove salt. The supernatant solution (or filtrate or both) and washings were combined, dialysed to remove $(NH_4)_2SO_4$ and concentrated to about 100 ml. Both materials were then dried from the frozen state, weighed and analysed. The preparations were dissolved separately in water to give about 1% (w/v) concentration, saturated with $(NH_4)_2SO_4$ at 60° and heated at this temperature for about 5 min. The material which was originally insoluble under these conditions again precipitated and the component which was earlier soluble in the hot saturated $(NH_4)_2SO_4$ remained in solution. The materials recovered after the second treatment with $(NH_4)_2SO_4$ showed no significant change in weight. The analytical figures for the two materials obtained are given in Table 1.

The fractionation experiment was repeated on the phenol-insoluble mucopolysaccharide obtained from another group A cyst fluid (no. 368). A similar separation into material which was insoluble or soluble in saturated $(NH_4)_2SO_4$ at 60° occurred. The analytical figures are given in Table 1.

Mucopolysaccharide materials obtained from two group O cyst fluids (nos. 338 and 324), from a group $A_1$B-active fluid (no. 117), a fluid obtained from a person belonging to group $A_2$B (no. 186) and from a $Le^a$ + 'non-secretor' person (cyst no. 238) were similarly separated into mucopolysaccharides which were either insoluble or soluble in saturated $(NH_4)_2SO_4$ at 60°. All substances were finally precipitated from water by ethanol, thoroughly dialysed at 1–3°, centrifuged at 30 000 *g* for 30 min. to remove traces of insoluble material, dried from the frozen state and analysed. The results are given in Table 1.

*Polydispersity of mucopolysaccharide preparations.* Material (about 1 g.), dissolved in phosphate buffer (*I* 0·2, pH 8·0) containing 0·15 M NaCl to yield a 1–2% (w/v) solution, was centrifuged for 5 hr. at 0–1° and 105 000 *g* (average value) in a Spinco (model L) preparative centrifuge. The contents of each of the centrifuge tubes were then divided into four approximately equal parts, starting from the top, and each consisting of the liquid contained in about a 1·8 cm. length of the centrifuge tube. The materials so recovered were separately dialysed to remove salts and dried from the frozen state. There was usually a small amount of the mucopolysaccharide deposited on the bottom of the centrifuge as a semi-transparent gel.

## RESULTS

The results of the experiments described above, and of many similar ones not described here, show that highly active preparations of the blood-group-specific mucopolysaccharides, obtained from dried ovarian-cyst fluid by extraction with 90% (w/w) phenol, can be separated in most instances into at least two mucopolysaccharide components, one of which is insoluble in saturated ammonium sulphate at 60°, and the other completely soluble under these conditions. The relative amount of each material finally recovered from the phenol-insoluble, water-soluble mucopolysaccharide preparation varies considerably according to the cyst fluid. Four cyst fluids, the total so far available to us, derived from 'secretors' belonging to group B, were all found to be soluble in saturated ammonium sulphate at 60°. The analytical figures obtained for these materials were, however, closely similar to those obtained for the other group-specific substances and will be described later.

In three instances, cyst fluids no. 277 (group O), no. 346 (group $A_1$) and no. 350 (group $Le^a$), the phenol-insoluble, water-soluble material recovered was found to be almost completely insoluble in saturated ammonium sulphate at 60°.

*Physical properties.* The mucopolysaccharides precipitated by hot saturated ammonium sulphate solution frequently separate as a thick visco-elastic mass which can be easily and completely recovered. Some mucopolysaccharides are, however, first thrown out of solution as light flocculent precipitates which rapidly become sticky, coalesce and are then readily collected from the surface of the saturated ammonium sulphate. The relative viscosity of the mucopolysaccharides recovered shows considerable variation (Table 1). Repeated treatment of the materials with hot ammonium sulphate under the conditions described, however, does not alter their viscosity. The values found for the relative viscosity of a 1% (w/v) solution of the substances in water at 20° show that the materials insoluble in hot ammonium sulphate possess, in every instance examined, a higher viscosity than that found for the materials soluble in saturated ammonium

Table 1. *Analytical values for the blood-group-specific mucopolysaccharides from human ovarian-cyst fluids*

An aqueous 2% (w/v) solution of the 90% phenol-insoluble material (*a*) from the dried cyst contents was saturated with ammonium sulphate and heated to 60° for 5 min. The material precipitated (*b*) and that (*c*) remaining soluble were recovered and analysed.

| Cyst fluid no. ... ... ... | 332 ($A_1$) | | | 368 ($A_1$) | | | 338 (H) | | | 324 (H) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (*a*) | (*b*) | (*c*) | (*a*) | (*b*) | (*c*) | (*a*) | (*b*) | (*c*) | (*a*) | (*b*) | (*c*) |
| Wt. of material (g.) | 3·0 | 2·1 | 0·7 | 4·5 | 3·0 | 1·2 | 2·8 | 1·5 | 1·2 | 2·5 | 1·5 | 0·7 |
| Nitrogen (%) | 4·7 | 5·3 | 4·4 | 4·9 | 5·0 | 4·4 | 6·3 | 6·0 | 3·9 | 5·9 | 6·4 | 4·7 |
| Fucose (%) | 19·3 | 18·4 | 20·6 | 17·0 | 16·1 | 17·6 | 18·0 | 19·4 | 22·7 | 17·0 | 17·2 | 18·3 |
| Hexosamine (%)* | 28·8 | 29·2 | 30·2 | 29·6 | 29·2 | 31·4 | 22·8 | 24·3 | 27·6 | 26·0 | 26·8 | 28·6 |
| Reducing sugar (%)† | 52·2 | 52·4 | 56·1 | 52·0 | 50·6 | 55·2 | 43·6 | 44·5 | 57·3 | 47·3 | 47·5 | 54·4 |
| Relative viscosity ($\eta$) | 2·39 | 4·28 | 1·80 | 4·32 | 6·80 | 1·96 | — | 5·37 | 2·11 | 12·65 | 4·40 | 2·38 |
| $[\alpha]_D$ in water (*c*, 1%) | +2° | +10° | −18° | — | — | — | —‡ | — | — | −34° | −28° | −36° |
| Serological activity ($\mu$g.)§ | 0·012‖ | 0·006‖ | 0·400‖ | 0·04‖ | 0·04‖ | 0·30‖ | 0·02¶ | 0·01¶ | 0·04¶ | 0·25¶ | 0·10¶ | 0·25¶ |
| | — | — | — | — | — | — | 8·0†† | 2·0†† | 62†† | 2·0†† | 2·0†† | 4·0†† |

| | 117 ($A_1B$) | | | 186 ($A_2B$) | | | 238 ($Le^a$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | (*a*) | (*b*) | (*c*) | (*a*) | (*b*) | (*c*) | (*a*) | (*b*) | (*c*) |
| Wt. of material (g.) | 0·9 | 0·6 | 0·2 | 20 | 10·2 | 5·2 | 2·1 | 1·2 | 0·7 |
| Nitrogen (%) | 5·3 | 5·4 | 4·6 | 4·7 | 5·3 | 4·1 | 4·8 | 4·9 | 4·4 |
| Fucose (%) | 17·9 | 18·5 | 17·3 | 17·9 | 20·0 | 22·2 | 13·1 | 13·2 | 13·2 |
| Hexosamine (%)* | 28·9 | 28·6 | 28·2 | 25·9 | 25·8 | 32·0 | 29·9 | 29·6 | 30·8 |
| Reducing sugar (%)† | 52·4 | 53·1 | 49·8 | 51·2 | 49·6 | 56·9 | 56·2 | 56·8 | 59·2 |
| Relative viscosity ($\eta$) | 4·28 | 4·61 | 2·14 | 2·64 | 3·45 | 2·03 | 3·92 | 4·88 | 2·74 |
| $[\alpha]_D$ in water (*c*, 1%) | −7° | +1° | −17° | −37° | −28° | −41° | −50° | −48° | −53° |
| Serological activity ($\mu$g.)§ | 0·02‖ | 0·01‖ | 0·30‖ | — | 0·05‖ | 0·20‖ | 0·003** | 0·003** | 0·006** |
| | — | — | — | 125†† | 125†† | Inactive†† | — | — | — |

* Expressed as glucosamine base after hydrolysis with 0·5 N-HCl for 16 hr. at 100°.
† Expressed as glucose after hydrolysis with 0·5 N-HCl for 16 hr. at 100°.
‡ Too opalescent for accurate measurement.
§ Minimum amount ($\mu$g.) giving complete inhibition of test dose of haemagglutinin.
‖ Titrated with human anti-A iso-agglutinin.
¶ Titrated with anti-H rabbit serum.
** Titrated with human anti-$Le^a$ serum Aalerud.
†† Titrated with human anti-H serum.

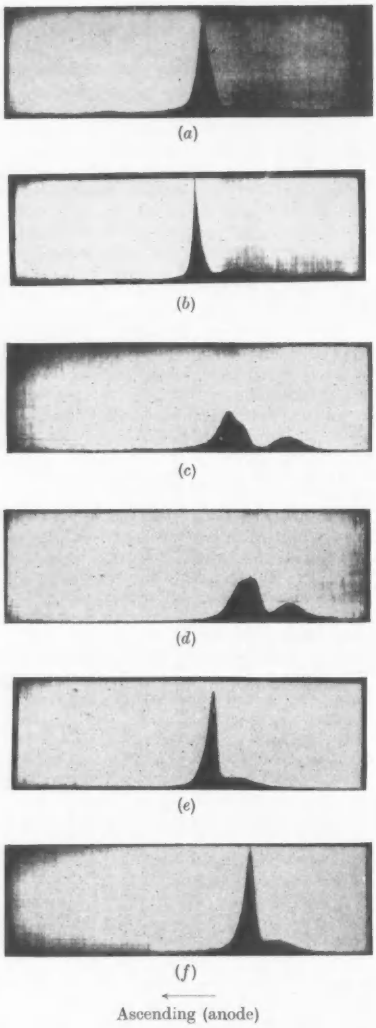


Fig. 1. Electrophoretic patterns of H-active mucopolysaccharides. Ascending boundaries in phosphate buffer, pH 8, $I$ 0·2, after 55 mA hr. Concn. 2% (w/v). Materials (*a*) (324) and (*b*) (277) are mucopolysaccharides insoluble in 90% phenol, soluble in water and insoluble in saturated $(NH_4)_2SO_4$ at 60°. Substance (*c*) is material from cyst substance 324, which is insoluble in 90% phenol and without further treatment. Material (*d*) is substance derived from 324, which was soluble in saturated $(NH_4)_2SO_4$ at 60°. Phenol-insoluble, water-insoluble mucopolysaccharide 324, was made soluble with pepsin at pH 1·9 and separated into materials insoluble (*e*) and soluble (*f*) in saturated $(NH_4)_2SO_4$ at 60°.

sulphate at 60° (Table 1), provided that the substances compared are obtained from the same cyst fluid.

Immediately after their precipitation from hot saturated ammonium sulphate, the mucopolysaccharides are insoluble in cold saturated ammonium sulphate solution, whereas after they have been freed from salt by dialysis and recovered they are soluble in cold saturated ammonium sulphate, as indeed they were before heating.

The optical rotation of the mucopolysaccharides insoluble in hot saturated ammonium sulphate are less laevorotatory than are the materials soluble under these conditions and obtained from the same phenol-insoluble, water-soluble cyst preparation (Table 1).

Electrophoretic examination of the materials obtained from the phenol-insoluble preparations shows that the materials insoluble in saturated ammonium sulphate at 60° are usually electrophoretically homogeneous (Fig. 1*a*, *b*), and this finding holds for the ammonium sulphate-insoluble mucopolysaccharides obtained from the phenol-insoluble water-soluble preparations which are themselves electrophoretically inhomogeneous (Fig. 1*c*). The materials soluble in hot saturated ammonium sulphate, however, frequently show some degree of electrophoretic heterogeneity (Fig. 1*d*), which is perhaps to be expected since the materials soluble in saturated ammonium sulphate at 60° might well contain, besides the serologically specific mucopolysaccharide, other materials, there in relatively small amounts only, which remain in the phenol-insoluble water-soluble preparations and are soluble under these conditions. In certain instances, however, both the ammonium sulphate-insoluble and -soluble materials are electrophoretically homogeneous.

Ultracentrifugal studies were made only with electrophoretically homogeneous mucopolysaccharide preparations and the materials proved to be, without exception, substantially homogeneous, although polydisperse (Fig. 2*a–d*).

The sedimentation coefficients of the ammonium sulphate-insoluble and -soluble components derived from cyst 368 (group $A_1$) were determined at various concentrations, and the values obtained were found to be strongly dependent on the concentration (Table 2). The intrinsic viscosity value for each material was determined by a linear extrapolation to zero concentration of the $\eta$ spec./$c$ values (Table 2) plotted against the concentration. The molecular weight was calculated from the sedimentation and viscosity data by means of the formula (cf. Schachman, 1957)

$$M = \frac{4690 \times (S_{20,w})^{\frac{3}{2}} \times (\eta)^{\frac{1}{2}}}{(1 - \bar{v}\delta)^{\frac{3}{2}}},$$

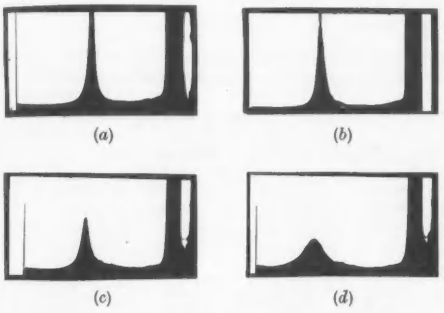


Fig. 2. Sedimentation diagrams of A- and H-active mucopolysaccharides. Substances (1%, w/v) in phosphate buffer at pH 8 ($I$ 0·2) containing 0·15M-NaCl. Centrifuging at 270 000 $g$. Substances ($a$), ($b$) and ($c$) are materials insoluble in 90% phenol, soluble in water and insoluble in saturated $(NH_4)_2SO_4$ at 60°, obtained respectively from cyst fluids no. 277 (H-active), 346 and 368 (A-active). ($a$) and ($b$) 135 min. and ($c$) 140 min. after reaching 270 000 $g$. Material ($d$) is insoluble in 90% phenol, soluble in saturated $(NH_4)_2SO_4$, from cyst fluid no. 368. Pattern after 140 min. at 270 000 $g$.

Table 2. *Physical properties of group A mucopolysaccharide from cyst fluid no.* 368

(1) Sedimentation coefficients, (2) intrinsic viscosity ($\eta$ spec./$c$) values at different concentrations, (3) partial specific volumes and (4) molecular weights for the mucopolysaccharide components ($a$) insoluble and ($b$) soluble in saturated ammonium sulphate at 60°. All measurements were made in phosphate buffer, pH 8·0 ($I$ 0·2), containing 0·15M-NaCl.

(1) Sedimentation coefficients

| Concn. (mg./ml.) | ($a$) | ($b$) |
|---|---|---|
| 10·0 | 5·56 | 5·42 |
| 7·5 | 6·36 | 5·95 |
| 5·0 | 7·05 | 6·57 |
| Infinite dilution | 8·55 | 7·73 |

(2) $\eta$ spec./$c$ values

| Concn. (mg./ml.) | ($a$) | ($b$) |
|---|---|---|
| 10·0 | 1·540 | 0·700 |
| 5·0 | 1·270 | 0·610 |
| 2·5 | 1·135 | 0·572 |
| 1·25 | 1·080 | — |
| Infinite dilution | 1·002 | 0·530 |

(3) Partial specific volumes

| Concn. (mg./ml.) | ($a$) | ($b$) |
|---|---|---|
| 10·0 | 0·625 | 0·625 |

(4) Molecular weight

| ($a$) | ($b$) |
|---|---|
| $5{\cdot}11 \times 10^5$ | $3{\cdot}19 \times 10^5$ |

where $M$ is the molecular weight, $S_{20,w}$ the sedimentation coefficient at infinite dilution, $[\eta]$ the intrinsic viscosity, $\bar{v}$ the partial specific volume and $\delta$ the density of the solution. The molecular weights obtained are given in Table 2.

The degree of polydispersity of a few mucopolysaccharide materials insoluble or soluble in saturated ammonium sulphate at 60° were determined, the simple procedure described in the Experimental section being used (see also Pusztai & Morgan, 1961). The results show that the material which was insoluble in saturated ammonium sulphate at 60°, and obtained from cyst fluid no. 368 (group $A_1$) was readily resolved into a range of molecular entities of different sizes in spite of the apparent homogeneity of the material when examined by the usual sedimentation procedure. The corresponding material which was soluble in saturated ammonium sulphate at 60°, and was obtained from the same cyst fluid, likewise yielded materials which showed a range of molecular sizes. The physical, chemical and serological data obtained for the different mucopolysaccharide fractions are given in Table 3. If the percentage recovery of each fraction obtained from these materials is plotted against the corresponding intrinsic viscosity value, a typical Gaussian curve for the molecular size distribution is obtained and shows that the majority of the mucopolysaccharide entities in each preparation are closely similar in size (Fig. 3).

Similar information on the approximate size distribution, in terms of intrinsic viscosity, in a preparation of group-specific mucopolysaccharide can also be obtained by careful fractionation with ethanol. Thus the component insoluble in saturated ammonium sulphate obtained from cyst fluid no. 368 was reprecipitated twice from 1% (w/v) solution between 50 and 55% ethanol concentration, and the resulting material was subjected to a similar fractionation in the preparative ultracentrifuge. The molecular size-distribution curve obtained showed a pronounced sharpening, resulting from the removal during fractionation of those mucopolysaccharide units having a smaller size than the average (Fig. 3).

*Chemical properties.* The analytical results given in Table 1 show that without exception the components insoluble in saturated ammonium sulphate at 60° contain more nitrogen than do the corresponding soluble materials, and that the latter, with the single exception of the substance recovered from the phenol-insoluble, water-soluble material obtained from cyst no. 117, $A_1B$, contain more carbohydrate than do the former. As the total amino sugar is closely similar for each pair of substances, it seems possible that components insoluble in saturated ammonium sulphate solution at 60° have a greater amount of amino acid per molecule of

Table 3. *Chemical, physical and serological properties of mucopolysaccharide preparations obtained from cyst fluid no.* 368 (*group A*)

Material insoluble in 90% phenol was separated into two mucopolysaccharide components: (*a*) insoluble and (*b*) soluble in saturated ammonium sulphate at 60°; each material was separated into five fractions by differential centrifuging at 105 000 *g* for 5 hr. at 0–2°.

| | Recovery | | N | Fucose | Amino sugar* | Reducing sugar* | Intrinsic viscosity | Serological activity: Haemagglutination-inhibition end point | Serological activity: Antibody N precipitated |
|---|---|---|---|---|---|---|---|---|---|
| | (mg.) | (%) | (%) | (%) | (%) | (%) | $\eta$ spec./$c$ | ($\mu$g.) | ($\mu$g.) |
| | | | | | (*a*) Insoluble | | | | |
| First fraction (top) | 64 | 6·3 | 4·7 | 15·8 | 28·6 | 49·4 | 0·637 | 0·08 | 99·0 |
| Second fraction | 190 | 18·4 | 4·8 | 15·9 | 29·0 | 50·2 | 0·876 | 0·04 | 101·6 |
| Third fraction | 233 | 22·6 | 4·9 | 16·0 | 29·4 | 50·8 | 0·920 | 0·04 | 103·0 |
| Fourth fraction (bottom) | 496 | 48·2 | 5·1 | 16·3 | 29·8 | 51·2 | 1·064 | 0·02 | 104·4 |
| Deposit | 47 | 4·5 | 5·4 | 15·8 | 28·2 | 50·0 | 1·310 | 0·02 | 104·4 |
| | | | | | (*b*) Soluble | | | | |
| First fraction (top) | 80 | 8·0 | 3·8 | 18·0 | 29·8 | 52·3 | 0·218 | 0·6 | 84·5 |
| Second fraction | 201 | 19·6 | 4·4 | 17·0 | 30·5 | 54·4 | 0·427 | 0·3 | 85·8 |
| Third fraction | 226 | 22·0 | 4·5 | 17·5 | 31·3 | 55·0 | 0·445 | 0·3 | 88·4 |
| Fourth fraction (bottom) | 503 | 48·8 | 4·4 | 17·4 | 31·8 | 55·6 | 0·535 | 0·15 | 89·8 |
| Deposit | 17 | 1·6 | 4·5 | 16·3 | 31·6 | 55·3 | 0·557 | 0·15 | 89·8 |

* After hydrolysis at 100° with 0·5 N-HCl for 18 hr. expressed as glucosamine base or as glucose.

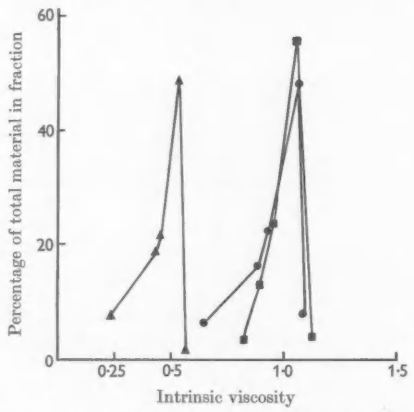


Fig. 3. Approximate size distribution of mucopolysaccharide preparations obtained from cyst fluid no. 368 (group $A_1$). The phenol-insoluble water-soluble material was separated into material soluble or insoluble in saturated $(NH_4)_2SO_4$ at 60°. Each material was centrifuged at 105 000 *g* and divided into five fractions (see text). A portion of the material insoluble in phenol, soluble in water, was also precipitated twice between 50 and 55% (v/v) of ethanol and similarly separated by centrifuging into five fractions. The percentage of total material centrifuged which was recovered in each fraction was plotted against the intrinsic viscosity of each fraction for material soluble (▲) or insoluble (●) in saturated $(NH_4)_2SO_4$ at 60° and for materials obtained from the ethanol-fractionated substance (■).

polysaccharide than have the soluble substances isolated from the same source. Some preliminary results for the amino acid composition of three pairs of substances are set out in Table 4.

*Serological properties.* Although the activity of individual preparations which possess the same specificity, for example, all of which belong to group A, show marked differences in quantitative serological behaviour, nevertheless the mucopolysaccharides insoluble in saturated ammonium sulphate which have A or H activity are found to be more active than the corresponding soluble substances obtained from the same cyst fluid (Table 1). However, the constant and significant difference found in the capacity of the two kinds of mucopolysaccharide material obtained from a single source to neutralize the natural human group-specific isoagglutinins is less pronounced when human or animal immune agglutinins, natural eel-serum or plant-seed agglutinins are employed as test reagents (Tables 5 and 6).

The amounts of antibody nitrogen precipitated from rabbit immune anti-A serum by the addition of increasing quantities of the original phenol-insoluble, water-soluble mucopolysaccharide and by the materials insoluble or soluble in ammonium sulphate separated from it, are shown in Figs. 4 (substance 332) and 5 (substance 186). In every instance examined so far, the material insoluble in saturated ammonium sulphate at 60° precipitates more antibody from the test serum than does the

Table 4. *Quantitative amino acid analyses of group-specific mucopolysaccharides*

Materials insoluble in 90% phenol isolated from cyst fluids were separated into mucopolysaccharides: (*a*) insoluble and (*b*) soluble in saturated $(NH_4)_2SO_4$ at 60°. Results are expressed as g. of amino acid/100 g. of mucopolysaccharide. Analysis of materials 332 (*a*) and (*b*) were kindly made by Dr Gabrielle Ellinger. Analyses of substances 324 (*a*) and (*b*), and 186 (*a*) and (*b*) were obtained by Dr K. Bailey to whom we wish to express our thanks.

| | A substance 332 | | H substance 324 | | Substance 186 | |
|---|---|---|---|---|---|---|
| Amino acid | (*a*) | (*b*) | (*a*) | (*b*) | (*a*) | (*b*) |
| Aspartic acid | 0·31 | 0·46 | 0·62 | 0·25 | 0·45 | 0·30 |
| Threonine | 4·05 | 2·90 | 5·55 | 3·90 | 3·60 | 1·90 |
| Serine | 2·16 | 1·57 | 1·61 | 2·11 | 2·20 | 1·13 |
| Glutamic acid | 0·72 | 0·82 | 1·02 | 0·53 | 0·59 | 0·39 |
| Proline | 2·04 | 1·62 | 2·42 | 1·79 | 1·50 | 1·66 |
| Glycine | 0·43 | 0·37 | 0·49 | 0·30 | 0·33 | 0·30 |
| Alanine | 0·92 | 0·83 | 1·00 | 0·65 | 0·64 | 0·71 |
| Valine | 0·59 | 0·41 | 0·80 | 0·46 | 0·55 | 0·33 |
| Methionine | 0·11 | 0·12 | 0·11 | — | — | — |
| Isoleucine | 0·41 | 0·31 | 0·49 | 0·41 | 0·31 | 0·15 |
| Leucine | 0·35 | 0·35 | 0·54 | 0·46 | 0·36 | 0·19 |
| Tyrosine | 0·15 | 0·08 | 0·23 | — | 0·15 | 0·32 |
| Phenylalanine | 0·29 | 0·26 | 0·27 | — | 0·20 | |
| Lysine | 0·35 | 0·27 | 0·76 | 0·71 | 0·77 | 0·33 |
| Histidine | 0·70 | 0·78 | 0·53 | 0·70 | 0·49 | 0·44 |
| Arginine | 0·50 | 0·44 | 0·61 | 0·27 | 0·71 | 0·38 |
| | 14·08 | 11·59 | 17·05 | 12·54 | 12·85 | 8·53 |

Table 5. *Haemagglutination-inhibition end points and activity ratios of blood-group A-specific mucopolysaccharides*

Materials (*a*) insoluble and (*b*) soluble in saturated $(NH_4)_2SO_4$ at 60° were titrated in inhibition tests with three to four agglutination doses of anti-A reagents of different origin and group A cells. Results are given as minimum amount (μg.) of substance giving inhibition of agglutination.

| | A substance from cyst fluid no. 332 | | | $A_2B$ substance from cyst fluid no. 186 | | |
|---|---|---|---|---|---|---|
| Anti-A reagent | (*a*) | (*b*) | (*b*)/(*a*) | (*a*) | (*b*) | (*b*)/(*a*) |
| Normal human serum (1:10)* | 0·006 | 0·4 | 66 | 0·25 | 8 | 32 |
| Immune human serum (1:50)* | 0·4 | 6 | 15 | 62 | 500 | 8 |
| Immune rabbit serum (1:200)* | 0·4 | 1·6 | 4 | 1 | 8 | 8 |
| *Vicia cracca* extract (1:30)* | 0·4 | 1·6 | 4 | 1 | 4 | 4 |
| *Dolichos biflorus* extract (1:20)* | 3 | 6 | 2 | 4 | 16 | 4 |

* Dilution of serum or 10% seed extract used as test dose of agglutinin.

Table 6. *Haemagglutination-inhibition end points and activity ratios of H-active mucopolysaccharides*

Materials (*a*) insoluble and (*b*) soluble in saturated $(NH_4)_2SO_4$ at 60° were titrated in inhibition tests with three to four agglutination doses of anti-H reagents of different origin and group O cells. Results are given as minimum amount (μg.) of substance giving inhibition of agglutination.

| | Substance from cyst fluid no. 186 ($A_2B$) | | | H substance from cyst fluid no. 338 | | |
|---|---|---|---|---|---|---|
| Anti-H reagent* | (*a*) | (*b*) | (*b*)/(*a*) | (*a*) | (*b*) | (*b*)/(*a*) |
| Immune human serum (1:100) | 125 | 500 | 4 | 2 | 62 | 31 |
| Rabbit immune serum (1:25) | 0·05 | 0·2 | 4 | 0·25 | 0·50 | 2 |
| Cattle serum (1:2) | 0·2 | 0·2 | 1 | 2 | 4 | 2 |
| Eel serum (1:50) | 0·8 | 0·8 | 1 | 2 | 8 | 4 |

* See footnote Table 5.

ammonium sulphate-soluble component obtained from the same source.

The serological activity of each fraction obtained from the ammonium sulphate-insoluble or -soluble components of A substance (cyst no. 368) after differential sedimentation at 105 000 *g* for 5 hr. was plotted against the intrinsic viscosity value of each material, the estimate of the serological activity being obtained from the measurement of the minimum amount of material required (*a*) to inhibit isoagglutination (Fig. 6) or (*b*) precipitate antibody nitrogen (Fig. 7).

The so-called Forssman activity (Schiff & Adelsberger, 1924) of this group A substance, and of the materials separated from it by ammonium sulphate treatment, was found to be the same within the limits of sensitivity of the test system.

*Action of pepsin on the mucopolysaccharides*

The consistently higher nitrogen content of the ammonium sulphate-insoluble mucopolysaccharide compared with that of the corresponding soluble material obtained from the same preparation of phenol-insoluble, water-soluble mucopolysaccharide made it expedient to establish that the additional nitrogen was a part of the macromolecule and not a co-existing impurity, and whether it could be removed without changing the physical and serological properties of the mucopolysaccharide. To obtain this evidence the mucopolysaccharide obtained from cyst fluid 186 (Table 1), which was insoluble in saturated ammonium sulphate at 60° after three treatments with this reagent, was digested with crystalline pepsin and the products were recovered. The mucopolysaccharide remaining insoluble in saturated ammonium sulphate at 60°, however, was found to be closely similar in chemical composition to the original material, and there was no significant decrease in the nitrogen content (Table 7). A number of changes in the starting material had taken place, however, the most obvious being the formation of a certain amount of mucopolysaccharide which was soluble in hot saturated ammonium sulphate (Table 7). The material was similar to, but not identical with, the ammonium sulphate-soluble material recovered from the original mucopolysaccharide preparation (Table 1, substance 186, *c*). As it was possible that some or all of the limited changes induced could

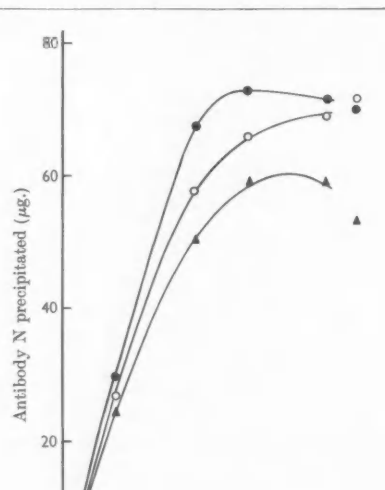


Fig. 4. Antibody N precipitated from antibody by the addition of increasing amounts of mucopolysaccharide. Substance no. 332 (group A), phenol-insoluble, water-soluble (○), and material derived from it which was insoluble (●) or soluble (▲) in saturated $(NH_4)_2SO_4$ at 60°.

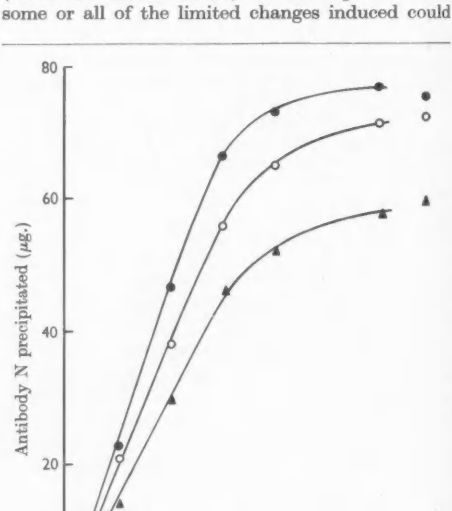


Fig. 5. Antibody N precipitated from antibody by the addition of increasing amounts of mucopolysaccharide. Substance no. 186 (group $A_2B$), phenol-insoluble, water-soluble (○), and material derived from it which was insoluble (●) or soluble (▲) in saturated $(NH_4)_2SO_4$ at 60°.

have been due to hydrolysis brought about by the citrate–hydrochloric acid buffer (pH 1·9) acting for 3 days at 37°, the following control experiment was made. A portion (200 mg.) of the mucopolysaccharide was incubated under the same conditions but without pepsin. The major part of the material was unchanged and retained its insolubility in saturated ammonium sulphate at 60°. There was formed, however, about 25 % of a mucopolysaccharide material which was soluble in saturated ammonium sulphate at 60° and was closely similar in properties to the soluble component found after peptic digestion (Table 7).

The mucopolysaccharide soluble in saturated ammonium sulphate which was obtained from the phenol-insoluble, water-soluble material recovered from cyst fluid no. 186 (Table 1, substance 186, *c*) was similarly incubated with pepsin. The analytical figures for the recovered material indicated (Table 8) that little change had occurred. The particular preparation of rabbit anti-H serum used to determine the H activity (cf. Annison & Morgan, 1950) showed that there was some loss in capacity to neutralize H agglutinins. However, other rabbit anti-H reagents showed that it was not always possible to demonstrate loss in H activity after the H substance had been incubated with pepsin.

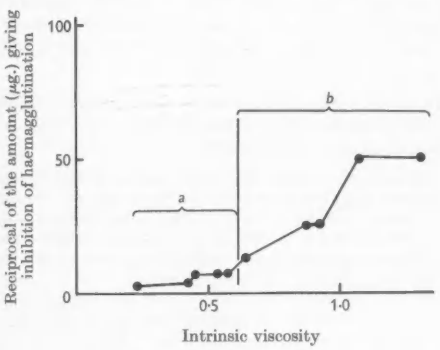


Fig. 6. Relation between serological activity and intrinsic viscosity of mucopolysaccharide preparations. Serological activity was determined as the minimum amount of substance (μg.) inhibiting three to four completely agglutinating doses of specific antibody. Titrations were made on phenol-insoluble, water-soluble substance obtained from cyst fluid no. 368 (group $A_1$) after the material had been separated into mucopolysaccharide which was (*a*) soluble or (*b*) insoluble in saturated $(NH_4)_2SO_4$ at 60°. Each substance was then separated into five fractions by centrifuging at 105 000 **g** (see text) and the intrinsic viscosity ($\eta$ spec./*c*) value of each fraction is plotted against the reciprocal of the serological activity of the fraction.

*Solubilization of a water-insoluble mucopolysaccharide gel with pepsin and treatment with saturated ammonium sulphate at 60°*

It has been known for a considerable time (Aminoff, Morgan & Watkins, 1950) that extraction of the freeze-dried contents of ovarian-cyst fluids and other secretions with 90 % (w/w) phenol gives a mucopolysaccharide which is insoluble in this solvent and is, in part, insoluble in water. These water-insoluble components are readily solubilized by pepsin and are then obtained in a suitable condition for treatment with hot saturated ammonium sulphate solution.

The phenol-insoluble, water-insoluble component of cyst fluid no. 324 (group O) was incubated with crystalline pepsin under the conditions given in Table 9. The gel dissolved rapidly and, after a few days' digestion, the solubilized mucopolysaccharide was isolated and finally precipitated from solution in water by the addition of ethanol up to 55 % (v/v) concentration. The analytical results (Table 9) show that after the solubilization with pepsin and fractionation with ammonium sulphate the mucopolysaccharide preparation is resolved into two muco-

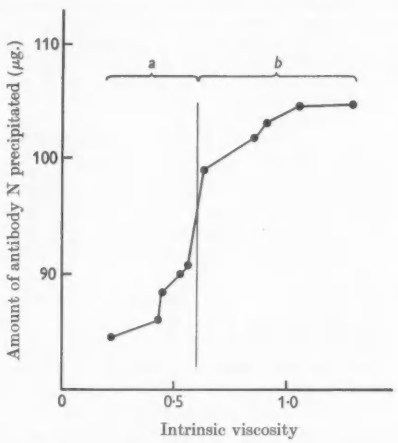


Fig. 7. Relation between serological activity and intrinsic viscosity of mucopolysaccharide preparations. Serological activity was determined as the maximum amount of antibody N (μg.) precipitated by the preparation in the presence of excess of antibody. Determinations were made on phenol-insoluble, water-soluble substance obtained from cyst fluid no. 368 (group $A_1$) after the material had been separated into mucopolysaccharide which was (*a*) soluble or (*b*) insoluble in saturated $(NH_4)_2SO_4$ at 60°. Each substance was then separated into five fractions by centrifuging at 105 000 **g** (see text) and the intrinsic viscosity ($\eta$ spec./*c*) value of each fraction was plotted against the serological activity of the fraction.

Table 7. *Action of pepsin on the specific mucopolysaccharide obtained from cyst fluid no.* 186 (*group* $A_2B$)

Mucopolysaccharide (2 g.), insoluble in saturated $(NH_4)_2SO_4$ at 60°, was incubated with crystalline pepsin (40 mg.) in 0·1 M-citrate–HCl buffer, pH 1·9, for 3 days at 37°. Control experiment (200 mg.): same mucopolysaccharide and buffer but without pepsin. Toluene was added as preservative. Material after digestion was dialysed and separated into mucopolysaccharide, (*a*) insoluble and (*b*) soluble in saturated $(NH_4)_2SO_4$ at 60°.

| | Original material | Substances recovered after incubation: With pepsin (*a*) | With pepsin (*b*) | Without pepsin (*a*) | Without pepsin (*b*) |
|---|---|---|---|---|---|
| Amount (g.) | — | 0·95 | 0·69 | 0·152 | 0·042 |
| Nitrogen (%) | 5·3 | 5·1 | 4·7 | 5·3 | 4·7 |
| Fucose (%) | 20·3 | 19·8 | 21·3 | 20·5 | 21·0 |
| Amino sugar (%)* | 25·1 | 25·7 | 27·2 | 25·8 | 26·9 |
| Reducing sugar (%)* | 50·0 | 50·6 | 54·2 | 49·6 | 53·8 |
| Relative viscosity ($\eta$) | 3·38 | 2·60 | 2·08 | 3·45 | 1·75 |
| $[\alpha]_D$ in water (*c*, 1%) | −30° | −22° | −26° | −28° | −26° |
| A activity ($\mu$g.)† | 1·0 | 1·0 | 4·0 | 1·0 | 4·0 |
| H activity ($\mu$g.)‡ | 0·2 | 1·6 | 3·2 | 0·2 | 0·4 |

* After hydrolysis at 100° with 0·5 N-HCl for 18 hr.
† Inhibition end point with human anti-A (α) serum.
‡ End point of inhibition with rabbit immune anti-H serum.

Table 8. *Action of pepsin on group-specific mucopolysaccharide obtained from cyst fluid no.* 186 (*group* $A_2B$)

Mucopolysaccharide (0·65 g.), soluble in saturated ammonium sulphate at 60°, was incubated with pepsin as described in Table 6. Material remained completely soluble in saturated $(NH_4)_2SO_4$ at 60° and, before analysis, was precipitated twice from 1% (w/v) solution in water between 45 and 50% (w/v) ethanol concentration.

| | Original material | Material after digestion |
|---|---|---|
| Nitrogen (%) | 4·2 | 3·9 |
| Fucose (%) | 21·6 | 22·2 |
| Amino sugar (%)* | 27·8 | 25·2 |
| Reducing sugar (%)* | 56·2 | 57·8 |
| Relative viscosity ($\eta$) | 1·96 | 1·50 |
| $[\alpha]_D$ in water (*c*, 1%) | −47° | −43° |
| A activity ($\mu$g.)† | 16 | 16 |
| H activity ($\mu$g.)‡ | 0·2 | 3 |

* See footnote Table 7.
† End point of inhibition with human anti-A (α) serum.
‡ End point of inhibition with rabbit immune anti-H serum.

polysaccharides, one of which is insoluble in hot saturated ammonium sulphate and the other soluble in this reagent. The substances are electrophoretically homogeneous at pH 8 (Fig. 1*e*, *f*) and are closely similar in those physical, chemical and serological properties examined to the mucopolysaccharides isolated from the material originally insoluble in 90% phenol, soluble in water and obtained from the same cyst fluid (Table 1, *b* and *c*). The procedure described has been applied, with similar results, to the paramucinous or 'trembling-jelly' type of ovarian-cyst contents.

*Isolation of blood-group-specific mucopolysaccharides from ovarian-cyst fluids without the use of phenol*

For reasons given by Morgan (1958) attempts were made to obtain blood-group-specific mucopolysaccharides from crude ovarian fluids without employing phenol at any stage in the isolation procedure. The large amount of unspecific protein in cyst fluids precludes the direct treatment with hot saturated ammonium sulphate, as described above, or indeed the use of methods which depend on the

Table 9. *Analytical figures for mucopolysaccharides obtained by the solubilization of water-insoluble mucopolysaccharide gel by pepsin*

Water-insoluble mucopolysaccharide gel from cyst fluid (no. 324) dissolved in 0·1 M-citrate–HCl buffer, pH 1·9, and incubated with 1% (w/w) crystalline pepsin for 3 days at 37°. Toluene was added as preservative. Digest was dialysed and separated into material (*a*) insoluble and (*b*) soluble in saturated $(NH_4)_2SO_4$ at 60°.

| | Original mucopolysaccharide gel | Gel solubilized with pepsin and separated into (*a*) | (*b*) |
|---|---|---|---|
| Amount (g.) | 3·9‡ | 1·9 | 1·8 |
| Nitrogen (%) | 7·7 | 6·1 | 5·0 |
| Fucose (%) | 9·7 | 16·0 | 17·8 |
| Amino sugar (%)* | 17·0 | 25·1 | 26·3 |
| Reducing sugar (%)* | 39·0 | 48·2 | 55·9 |
| Relative viscosity ($\eta$) | — | 3·52 | 1·79 |
| $[\alpha]_D$ in water (*c*, 1%) | — | −29° | −38° |
| H activity ($\mu$g.)† | — | 0·05 | 0·10 |

* See footnote Table 7.
† End point of inhibition with rabbit immune anti-H serum.
‡ Dry weight.

simple denaturation of the protein by heat or organic solvents, because any procedure involving the removal of large amounts of protein by precipitation also removes by entrainment and unspecific binding considerable amounts of mucopolysaccharide. A small amount of mucopolysaccharide which is soluble in saturated ammonium sulphate can usually be recovered from cyst fluids which are suitably diluted and saturated with this salt, and heated to 60°, thus demonstrating that mucopolysaccharides soluble under these conditions and isolated by the procedures described earlier in this paper exist in native cyst fluids. The method is, however, of limited practical importance.

Table 10. *Digestion of native cyst fluid with pepsin to recover mucopolysaccharide without the use of* 90% *phenol*

Native cyst fluid (no. 324, group O), 800 ml., was digested at pH 1·9 in 0·1M-citrate–HCl buffer with 50 mg. of crystalline pepsin. Toluene was added as preservative. Material was dialysed, saturated with $(NH_4)_2SO_4$ and heated at 60° for 5 min. Mucopolysaccharide materials (*a*) insoluble and (*b*) soluble, under these conditions, were recovered.

| | Mucopolysaccharide | |
|---|---|---|
| | (*a*) | (*b*) |
| Amount (g.) | 7·5 | 0·6 |
| Nitrogen (%) | 10·8 | 8·5 |
| Fucose (%) | 8·2 | 17·7 |
| Amino sugar (%)* | 12·0 | 25·8 |
| Reducing sugar (%)* | 24·4 | 50·2 |

* See footnote Table 7.

Table 11. *Fractionation of a pepsin digest of ovarian-cyst fluid*

(*a*) Material (6·8 g.) insoluble in saturated $(NH_4)_2SO_4$ (Table 10, substance *a*) was extracted with dimethylsulphoxide (800 ml.) and the resulting solution fractionated with ethanol. Material was recovered between 20 and 60% of ethanol (4·6 g.). Re-extracted with dimethylsulphoxide and recovered between 30 and 50% (w/v) ethanol concentration. Material was dissolved in water and fractionated with ethanol between 45 and 50% concentrations.

(*b*) Material (0·6 g.) soluble in $(NH_4)_2SO_4$ at 60° (Table 10, substance *b*) was repeatedly fractionated with ethanol between 50 and 55% concentrations.

| | Mucopolysaccharide | |
|---|---|---|
| | (*a*) | (*b*) |
| Nitrogen (%) | 6·0 | 5·0 |
| Fucose (%) | 17·4 | 17·5 |
| Amino sugar (%)* | 24·3 | 27·0 |
| Reducing sugar (%)* | 49·1 | 52·0 |
| Relative viscosity ($\eta$) | 5·24 | 1·79 |
| $[\alpha]_D$ in water (*c*, 1%) | −30° | −38° |
| H activity ($\mu$g.)† | 0·05 | 0·10 |

* See footnote Table 7.
† Titrated with rabbit immune anti-H serum.

Digestion of the cyst fluid with pepsin as a first stage in the isolation procedure will largely overcome the co-precipitation of mucopolysaccharide and protein. Thus native cyst fluid digested with pepsin, dialysed and the mucopolysaccharide components separated into materials insoluble and soluble in saturated ammonium sulphate at 60°, gave mucopolysaccharides which, in most instances, were contaminated with extraneous nitrogenous material (Table 10), most probably undigested protein and indiffusible hydrolysis products. The nitrogen values were, however, reduced by extracting the dry materials with dimethylsulphoxide and fractionation of the dissolved material with ethanol (Table 11).

The results show the materials obtained without the use of phenol are closely similar to those recovered from the same cyst fluid by the phenol-extraction procedure (Table 1).

## DISCUSSION

Evidence that it is possible to obtain from the secretions of a single individual at least two blood-group-specific mucopolysaccharides which differ in their physical, chemical and serological properties was brought forward by Gibbons & Morgan (1954), who isolated from an ovarian-cyst fluid obtained from a group B person, two mucopolysaccharides, designated B and B′ substances, that differed in their capacity to react with normal human anti-B ($\beta$) agglutinins, but which nevertheless were indistinguishable in their reactions with immune anti-B agglutinins of human or animal origin. The mucopolysaccharides differed in their nitrogen content and in their relative viscosities and optical rotation, but were in many other properties closely similar. These findings seemed to indicate that at least two forms of a blood-group-specific mucopolysaccharide could co-exist in the secretions of a single individual. Attempts to show at that time the presence of more than one kind of specific blood-group substance in the cyst fluids of other persons were not successful, and the results obtained with that particular B secretion were believed to be somewhat exceptional. It was considered unlikely, however, that the B′ substance was an artifact arising from the method of preparation since it was observed that the B substance was not demonstrably altered and changed into B′ substance under any of the conditions to which it was subjected during isolation.

It has now been found that if preparations of blood-group-specific mucopolysaccharide obtained by the extraction of freeze-dried ovarian-cyst fluids with 90% phenol (Gibbons *et al.* 1955) are dissolved in water, saturated with ammonium sulphate and heated to 60° for a few minutes, a mucopolysaccharide material is frequently precipitated from solu-

tion. The amount of mucopolysaccharide insoluble under these conditions varies considerably from one cyst fluid to the next and whereas, in some instances, about 95 % of the starting material is found to be insoluble, with other preparations such as the phenol-insoluble, water-soluble materials from H-active cyst fluid no. 278 (Morgan, 1959*b*), and all the B-active materials so far examined, 95 % or more of the phenol-insoluble, water-soluble substance remains soluble in the hot saturated ammonium sulphate. Most preparations of mucopolysaccharide obtained by phenol extraction, however, yield between a half and two-thirds of their weight as material insoluble in saturated ammonium sulphate at 60°, the remaining part being completely soluble in this reagent. After the first treatment with ammonium sulphate there is no evidence of change in weight or demonstrable change in properties if the isolated mucopolysaccharides are repeatedly exposed to saturated ammonium sulphate under the conditions described. It seems probable therefore that each kind of mucopolysaccharide exists as such in the native secretion.

Apart from the property of being either soluble or insoluble in saturated ammonium sulphate solution at 60°, the two mucopolysaccharides can be clearly differentiated in other of their properties, physical, chemical and serological. A 1 % solution in water or sodium chloride solution of the substance insoluble in saturated ammonium sulphate at 60° usually has a higher viscosity than that shown by the ammonium sulphate-soluble material obtained from the same cyst fluid. The results of the experiments which separate mucopolysaccharides into sizes by centrifuging indicate that within a family of similar molecules obtained from the same cyst fluid, the smallest size molecules in the material insoluble in saturated ammonium sulphate are apparently larger than the largest molecules in the material soluble in this reagent. Similarly, the optical rotations of the ammonium sulphate-insoluble preparations are much less laevorotatory than are those of the soluble materials, a finding which is in agreement with the conclusions of Schellman (1958), who considers that for molecules of similar kind, the optical rotation is determined by the folding and compactness of the molecule: the greater the folding and compactness, the less the laevorotation.

The physical measurements, especially the sedimentation constants, found for the ammonium sulphate-insoluble and -soluble materials, indicate a considerable difference in the molecular size of the two mucopolysaccharide preparations, and this fact may account for the inhomogeneity observed earlier for some carefully purified specimens of the specific substances. It seemed improbable at that time that the contaminant was protein as, in most instances, the materials which showed unsatisfactory physical behaviour possessed a lower nitrogen content than did the materials which were apparently homogeneous. The contaminant, however, could have been a substance of closely similar composition and structure which had an appreciably lower nitrogen content. Treatment of some of these early preparations with hot saturated ammonium sulphate has now shown that they can be resolved into at least two mucopolysaccharide components.

The molecular weights determined for the ammonium sulphate-insoluble and -soluble components of mucopolysaccharide no. 368 indicate that the average molecular size of the former component (511 000) is considerably larger than that found for the soluble component (319 000). The results obtained earlier (Pusztai & Morgan, 1960, 1961) for a similar pair of mucopolysaccharides, both of which, however, contained a sialic acid component, gave values of 237 000 and 107 000, which fall into line with the results now obtained with a neutral blood-group-specific mucopolysaccharide.

It seems possible that the separation of blood-group substances that are closely similar in chemical composition, into two materials, largely depends on the size of the component macromolecules. The molecules which have a size larger than a certain limiting value will be precipitated by hot ammonium sulphate, whereas all the smaller ones will remain in solution. This suggestion, however, although supported by the values found for the intrinsic viscosity of the two kinds of material obtained from the same cyst fluid, does not seem to hold when materials from different cysts are compared. The material soluble in saturated ammonium sulphate obtained from cyst fluid no. 368, for example, is larger (319 000) than is the material (237 000) insoluble in the same reagent, obtained from cyst fluid no. 350. A more extensive examination of further pairs of materials must be made before a final conclusion can be reached on the relationship between molecular size and the property of solubility or insolubility in saturated ammonium sulphate at 60°. The influence of the shape or compactness of the macromolecule on the solubility behaviour in saturated ammonium sulphate at 60° is not known.

Chemically, the two kinds of mucopolysaccharide preparation are similar, but not identical, and it is mostly found that the ammonium sulphate-insoluble component has a higher nitrogen content than has the soluble mucopolysaccharide. A quantitative determination of the individual amino acids in three pairs of specific mucopolysaccharides, each pair isolated from a single cyst fluid, showed that the components insoluble in saturated ammonium sulphate at 60° had appreciably more amino acid per 100 g. of mucopolysaccharide than did the corresponding soluble components, thus indicating that

the insoluble materials had more amino acid built into their macromolecular structure. If, however, the amount of an individual amino acid is considered, this conclusion does not necessarily hold. Thus the amount of the two acidic amino acids is larger (1·28 g./100 g.) in the ammonium sulphate-soluble component of A substance no. 332 than in the corresponding insoluble mucopolysaccharide (1·03 g./100 g.). Similarly, the amount of serine (1·61 g./100 g.) is significantly less in the ammonium sulphate-insoluble component of H substance from cyst fluid no. 324 than in the corresponding ammonium sulphate-soluble material (2·11 g./100 g.). The same applies to proline (1·50 and 1·66 g./100 g.) in the corresponding preparations from cyst fluid 186 ($A_2B$). These preliminary results indicate that although the general pattern of the amino acid-containing units in the mucopolysaccharide is similar, there are nevertheless apparently significant differences in the amounts of total and individual amino acids in each substance. This finding is being more closely examined with other highly purified materials. The analyses obtained on the materials, however, confirm the earlier findings (Aminoff *et al.* 1950; Morgan, 1954–55; Gibbons & Morgan, 1954; Carsten & Kabat, 1956) that there is a minimum of 11 or so amino acids present in the blood-group-specific mucopolysaccharides and that the hydroxyamino acids and proline make up rather more than half the total amino acids. It seems that phenylalanine, and perhaps other amino acids, must be included as components, although they are present in variable and in rather small amounts. Nevertheless, they occur in what are believed to be highly purified specimens of the specific mucopolysaccharides. The irregularities found in the amount and composition of the amino acids in the mucopolysaccharide preparations makes it advisable to consider whether such differences, if confirmed, can also contribute to the solubility behaviour of the blood-group-specific mucopolysaccharides in hot saturated ammonium sulphate.

Further and most interesting differences are also found in the serological behaviour of the two kinds of material. The mucopolysaccharides which are insoluble in saturated ammonium sulphate at 60° have a greater capacity to neutralize natural isoagglutinin than have the corresponding materials which are soluble, and this behaviour holds, irrespective of the group specificity within the ABO system; it also holds for the corresponding $Le^a$-specific mucopolysaccharides. The difference in the capacity of the two kinds of mucopolysaccharides to inhibit haemagglutinin is most pronounced when the naturally occurring antibodies in human serum are used as test reagents. Immune human and rabbit agglutinins are less satisfactory reagents to demonstrate this difference and plant-seed agglutinins sometimes fail to distinguish serologically between materials which are insoluble or soluble in saturated ammonium sulphate at 60°.

Examination by the serological precipitation technique of Heidelberger & MacPherson (1943) of the two forms of A-specific mucopolysaccharide obtained from the same cyst fluid shows that the mucopolysaccharide insoluble in ammonium sulphate precipitates more antibody nitrogen from a given anti-A serum than does that which is soluble in this reagent. This confirms the conclusion drawn from the results obtained by the haemagglutination-inhibition test, that the material insoluble in hot saturated ammonium sulphate is more active than the soluble component. The behaviour of mucopolysaccharide components isolated from each of these materials by high-speed centrifuging and selected according to their intrinsic viscosity values further indicates that the larger macromolecules precipitate a greater amount of antibody nitrogen from an antiserum than do the smaller-sized units. The results taken together therefore suggest that the specifically reactive structures of the larger mucopolysaccharide molecules combine with the receptor sites on the antibody molecules more satisfactorily than do those of the smaller molecules, and that there is a fraction of antibody which is not precipitated from the serum by this latter mucopolysaccharide material. One might tentatively assume that the quantitative difference in serological behaviour is, to a considerable extent, also determined by a secondary macromolecular structure and that the ultimate conformation of the larger of the macromolecules is better suited for serological reaction. Evidence in agreement with this conclusion and based on the results of ultrasonic treatment (Morgan, 1959*c*) and the action of crystalline papain and ficin (Pusztai & Morgan, 1958; Pusztai, 1960) on the specific mucopolysaccharides, have already been obtained.

After prolonged peptic digestion at a moderately high $H^+$ ion concentration, a considerable part of the mucopolysaccharide which is insoluble in saturated ammonium sulphate at 60° remains insoluble in this reagent and is, apparently, very resistant to modification by pepsin at pH 1·9. Nevertheless, after peptic digestion a part of the material becomes soluble in hot saturated ammonium sulphate. In the absence of pepsin, however, it is shown that the acid conditions alone bring about the formation of some material which is soluble, and it must be concluded that peptic digestion, under conditions of high $H^+$ ion concentration, brings about a limited and possibly significant amount of degradation.

The relative resistance of the specific substance to peptic hydrolysis under the conditions described allows a method to be elaborated which permits the

group-specific mucopolysaccharides to be isolated from ovarian-cyst fluids, and from other similar secretions not described in this paper, such as saliva, gastric juice and tissue digests, without the use of phenol. The water-insoluble mucopolysaccharide which frequently remains when phenol is used to isolate and purify the group substance does not arise when digestion of the native materials with pepsin and subsequent treatment with hot saturated ammonium sulphate is employed. The results of this treatment support the conclusion, reached earlier, that the water-soluble mucopolysaccharide which is insoluble in 90% phenol is not modified during isolation from the native cyst fluid. The materials recovered through the action of pepsin, followed by treatment with saturated ammonium sulphate at 60°, are closely similar to the phenol-insoluble, water-soluble materials originally described, provided that materials from the same source are used for each procedure employed. Of the two methods, extraction with 90% phenol has the advantage of simplicity, but the disadvantage of giving a lower yield owing to the recovery of the larger molecules as water-insoluble gels. On the other hand, the preliminary incubation of the tissue fluid with pepsin is a longer process, and induces a certain limited amount of degradation of the mucopolysaccharide by the acid conditions of digestion, but water-insoluble materials are not obtained. The method can be used for the solubilization of paramucinous cyst contents and tissues which are difficult or impossible to handle successfully by treatment with liquid phenol alone. It is to be observed, however, that secretions which contain sialomucopolysaccharide should not be exposed to digestion with pepsin as, at the acid reaction employed, the sialic acid component is readily eliminated (Pusztai & Morgan, 1960, 1961).

## SUMMARY

1. Blood-group A-, H- and Le$^a$-specific mucopolysaccharides, isolated from human ovarian-cyst fluids by extracting the dry cyst contents with 90% phenol, can be separated in many instances into two closely similar mucopolysaccharide materials by heating in saturated ammonium sulphate solution at 60° for a few minutes. A part of the mucopolysaccharide preparation is precipitated and a part remains in solution. The group B-active mucopolysaccharides insoluble in 90% phenol so far examined are almost completely soluble in saturated ammonium sulphate at 60°.

2. The mucopolysaccharides insoluble or soluble in saturated ammonium sulphate at 60° possess the same qualitative composition, but differ in certain physical and chemical properties. The materials insoluble under these conditions have a greater content of amino acid, are more viscous and have a larger molecular weight than have the materials soluble in ammonium sulphate, provided that the materials compared are obtained from the same cyst fluid.

3. The mucopolysaccharides insoluble in saturated ammonium sulphate at 60° are more active in haemagglutination-inhibition tests than are the ammonium sulphate-soluble materials. Group A-specific mucopolysaccharides, which are insoluble under these conditions, precipitate more antibody nitrogen from a specific antiserum than do the corresponding materials which are soluble in saturated ammonium sulphate at 60°.

4. Digestion of native cyst fluids or water-insoluble paramucinous gels with pepsin at pH 1·9, followed by treatment with saturated ammonium sulphate at 60°, allows blood-group substances to be recovered without the use of phenol and provides an alternative method for isolating these materials.

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# The Reaction between Elastase and Elastic Tissue

## 4. SOLUBLE ELASTINS*

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Hall (1951) reported that, if the ratio of solution to solid was raised sufficiently, elastin could be made to pass into solution by prolonged treatment with boiling 40% urea solution. Adair, Davis & Partridge (1951) also reported the dissolution of elastin by dilute oxalic acid. Before this, elastin was considered to be an exceptionally stable protein, in that the normal solvents were without effect on it. Horbaczewski (1882) described a material to which he gave the name hemielastin, derived from native elastic tissue by treatment with hydrochloric acid and pepsin, and further details of a similar material (elastose) were given by Richards & Gies (1902). These authors indicated that the soluble material obtained from elastin exhibited decreased solubility at elevated temperatures. This particular property was further studied by Adair *et al.* (1951), who showed that the phenomenon was due to the separation of the solution into protein-rich and protein-poor fractions, presumably by the production of coacervates between the molecules of the soluble elastin. Bowen (1953), by ultracentrifuge and diffusion studies, revealed that soluble elastin prepared with urea consisted of polydisperse protein of relatively high molecular weight. At least two molecular species appeared to be present and the results were consistent with the existence of an equilibrium between the two. Partridge, Davis & Adair (1955) separated the products of oxalic acid action into two main components and characterized their physical and chemical properties (Partridge & Davis, 1955). The high-molecular-weight protein ($\alpha$-elastin) was polydisperse and capable of forming coacervates resulting in a two-phase system similar to that observed by Bowen. Low-molecular-weight material ($\beta$-elastin) did not form coacervates on warming and was monodisperse. It is significant that the two proteins were reported as having almost identical amino acid analyses, and the terminal amino and carboxyl group content of the two species was consistent with the view that if $\alpha$-elastin were built up from $\beta$-elastin residues, then the latter must be joined through unknown cross-linkages. These same authors showed that such linkages could not be disulphide in nature.

Wood (1958) obtained soluble elastin by dissolution in a variety of solvents and showed that a material, having many of the properties of native elastin, could be reconstituted by maintaining the soluble material in a coacervated state at an elevated temperature for long periods.

The present paper reports new methods of preparing $\alpha$-elastin and additional observations on its properties. The effect of detergents throws light on the phenomenon of coacervation and the results of studies on the proteolytic action of elastase on $\alpha$-elastin may contribute to the general theory of proteolysis.

* The paper by Hall & Czerkawski (1959) is to be regarded as Part 3.

## MATERIALS AND METHODS

*Elastin and elastase.* A standard elastin powder derived from ox ligamentum nuchae by the methods described by Hall (1955, 1957) was used as starting material for all the preparations of soluble elastin. The enzyme employed was the electrophoretically pure preparation of elastase described by Hall & Czerkawski (1959).

*Measurement of the extent of coacervation.* The nature of the soluble elastin produced by the various methods was determined by the degree of coacervation obtained on warming. In certain key experiments, a constant-temperature cell housing (constructed in these laboratories) was used in a Unicam SP. 500 spectrophotometer to control the temperature to within 0·2° over the range 20–40°. Extinction was measured at 400 mμ and a pinhole attachment was fitted in front of the shutter opening of the photocell housing to minimize the effect of non-specific scattering. When expedient, however, especially in serial experiments, the extent of coacervation was measured in a more qualitative fashion. The protein solutions were brought to the required temperature in matched ½ in. test tubes in a water bath. The turbidity was measured in an EEL photoelectric colorimeter calibrated directly in extinction units. With a little practice, any individual reading could be completed before the temperature dropped by more than 0·5°.

*Measurement of the rate of proteolysis.* Three methods were used: two measured the extent of proteolysis in terms of amino group release and the third utilized trichloroacetic acid precipitation, the extent of which was taken as a measure of the molecular size of the protein.

In the first method (Spies, 1952) a copper complex which absorbed strongly at 230 mμ was formed at pH 9·3. The magnitude of absorption depends on the number of amino acid residues in the peptide or protein when these are of relatively low molecular weight, but with larger protein molecules the extinction is proportional to the number of free amino groups. The second method used the reaction with ninhydrin (Moore & Stein, 1948), alanine being employed as standard.

In the third method, 2 ml. of 30% (w/v) trichloroacetic acid was added to 2 ml. of protein solution in a centrifuge tube, and the mixture was kept at room temperature for 10 min. After centrifuging, 2 ml. of the supernatant was withdrawn and the non precipitable protein determined by means of the biuret reaction. With dilute protein solutions, the supernatant was poured off, the tubes were inverted to drain, 2 ml. of borate buffer (pH 8·5) was added to dissolve the precipitate, and the biuret reagent added directly to this.

### *Preparation of soluble elastin*

Several methods are now available for the preparation of α-elastin, in addition to the methods with oxalic acid and urea.

*Alkaline hydrolysis.* Although treatment with 0·1 N-alkali at 100° has been used by several workers as a method for the purification of elastin, Wood (1952) has shown that a sufficiently prolonged treatment with alkali even at lower temperatures results in the dissolution of the elastin. The solution so obtained consists in part of α-elastin.

Standard elastin powder (0·5 g.) was suspended in 50 ml. of 4 N-NaOH and incubated at 37°. Dissolution was complete in 20 hr., and the curve relating the amount of protein dissolved to the period of incubation was sigmoid and resembled similar curves relating to the solubilization of elastin by elastase. The product was a yellow solution which, when neutralized, dialysed and then brought to pH 4, had the power of coacervation. No attempts were made to fractionate this material since the presence of an appreciable amount of free amino groups indicated that the partial hydrolysis resulting from the action of alkali had degraded a relatively high proportion of the α-elastin.

*Treatment with strong mineral acid.* The action of strong acids for short periods was as effective as that of weak alkalis for longer periods.

Elastin (10 g.) was added with rapid stirring to 254 ml. of 80% (w/v) $H_2SO_4$ at 95° and stirred until most of the solid dissolved (3–4 min.). The solution was poured into 2750 ml. of cold water and neutralized with conc. aq. $NH_3$ solution. The pH was adjusted to 4 with dilute $H_2SO_4$ and the solution warmed to a temperature of 50°. The coacervated protein separated in the form of a brownish yellow, viscous mass, which readily adhered to a glass rod. The protein was then dissolved in 50 ml. of water; the solution was filtered to remove small amounts of unattacked elastin, dialysed against frequent changes of distilled water for two days, and freeze-dried to give 5·5 g. of a very soluble powder having all the properties of α-elastin.

After neutralization, the $(NH_4)_2SO_4$ concentration was approximately equivalent to the addition of 0·2 vol. of a saturated solution of the salt. The protein was used extensively in subsequent experiments because of the ease of preparation and because it exhibited a high degree of chemical homogeneity. Fractional precipitation with trichloroacetic acid showed that material precipitated by the addition of an equal volume of 5–40% (w/v) trichloroacetic acid had a constant amino group content of 32 moles/105 g. of protein. These fractions comprised more than 90% of the total protein.

*Hydrolysis by ethanol.* Partridge *et al.* (1955) observed that solubilization of elastin with oxalic acid is accompanied by the liberation of amino and carboxyl groups in about equal numbers and therefore deduced that the process consisted essentially of a hydrolytic reaction involving peptide bonds. As the protein can be dissolved under relatively mild conditions in the presence of alkali, it might be expected that elastin is stabilized in the solid state by unknown but highly significant bonds. Ester linkages have many of the properties which are necessary to produce the observed stability and the truth of such a hypothesis was tested by recourse to hydrolysis with hot acidified ethanol.

Gaseous HCl was passed into 100 ml. of absolute ethanol to render it 0·5 N with respect to the acid. Elastin powder (2·5 g.) was suspended in this solution and the mixture boiled under reflux for 3 hr., after which nearly all the elastin had passed into solution. The residue was removed by filtration while hot, and the filtrate allowed to cool to 10°. After 1 hr. at this temperature a precipitate formed which was removed by filtration, dissolved in water and freeze-dried to give 0·8 g. of protein (designated AP). The cool ethanolic supernatant was treated with 1·5 vol. of ether with the resulting formation of a bulky precipitate, which was dissolved in water, dialysed and freeze-dried to give a second product (0·6 g.; designated EP). Both proteins were strongly esterified (AP 48 moles of ethoxy groups/$10^5$ g. of protein, and EP 56 moles) and showed maximum coacerva-

tion at pH 9. Mild alkali treatment at room temperature resulted in a shift of this maximum to pH 4.

*Action of pepsin.* Horbaczewski (1882) prepared soluble elastin by the action of pepsin on elastin. During the present investigation pepsin dissolved solid elastin at pH 2, but the activity–time curve was unlike that with elastase at pH 8·5. The rate of dissolution at pH 2 is about one-fifteenth of that with an identical amount of elastase at pH 8·5. Nevertheless, the product can be made to coacervate at pH 5.

Elastin powder (1·0 g.) was incubated at 3° in 200 ml. of 0·1 M-acetate–HCl buffer (pH 2·0) containing 50 $\mu$g. of crystalline pepsin/ml. After 1 hr. the pH was raised to 7·0 and the mixture heated at 100° for 4 min. The unattacked solid was rather gelatinous and was removed by filtration in the presence of cellulose powder. The volume was made up to 330 ml. with water and 0·2 vol. of saturated $(NH_4)_2SO_4$ solution was added. The precipitate was then dialysed and freeze-dried to give 0·5 g. of powder.

*Controlled elastolysis.* The reaction between elastin and elastase takes place in two consecutive stages (Hall & Czerkawski, 1961). During the first of these, α-elastin is produced and thereafter this intermediate is converted into lower-molecular-weight material. The second half of the overall reaction can be inhibited by sodium dodecyl sulphate, in which case the α-elastin content of the system builds up.

Elastin (5 g.) was suspended in 380 ml. of borate buffer, pH 8·5; to this 0·5 g. of sodium dodecyl sulphate was added and the mixture kept at 37° for 1 hr. It was then chilled to 4° and mixed with 20 ml. of an elastase solution containing 0·2 mg. of pure elastase/ml. (Hall & Czerkawski, 1959) in borate buffer at the same pH. After 30 min. at 4° the mixture was incubated at 37° until dissolution was nearly complete (4–5 hr.) and heated at 100° for 4 min. to inactivate the enzyme.

The solution was dialysed for 24 hr. against running tap water to remove the greater part of the detergent, chilled to 4° and treated with 0·2 vol. of a saturated solution of $(NH_4)_2SO_4$. After 2 hr. the precipitate was separated by centrifuging and redissolved in 100 ml. of water. More detergent crystallized out on standing at −5°. The protein solution was then dialysed further, first against 20% $NH_4HCO_3$, water and aqueous 30% acetone and then against distilled water at 37°. The freeze-dried protein was a yellowish white powder (yield 4·2 g.).

## RESULTS

### *Conditions most suitable for optimum coacervation*

Coacervation is a function of pH, temperature, ionic strength and the concentration of the protein. Partridge *et al.* (1955) investigated the variation of this phenomenon with pH and ionic strength by noting the temperature at which the coacervate was either just noticeable or had just disappeared. Here the problem has been approached in a different fashion. Into each of a series of matched tubes were placed 2 ml. of a solution of soluble elastin, 2 ml. of buffer solution of a suitable pH and 4 ml. of water. The final protein concentration was 0·2 mg./ml. These mixtures were then heated at various temperatures and the turbidity was determined. The results of a typical experiment are shown in Fig. 1*a*. If an arbitrary line is drawn horizontally at any given value for extinction it cuts the family of curves in a number of points. This enables one to relate the temperature at which the degree of coacervation equals a given value against pH (Fig. 1*b*). Such results can then be compared directly with those of Partridge *et al.* (1955), and it can be concluded that there is a minimum temperature at finite pH, at all ionic strengths, but that the curves become more asymmetrical at higher ionic strengths until one side of the curve is almost lost. The optimum pH increases with temperature and decreases with ionic strength. If the pH, ionic strength and temperature are kept constant, the amount of coacervate increases steadily but not linearly with initial protein concentration.

### *Effect of detergents on coacervation*

Since the phenomenon of coacervation consists in the interaction between molecules and their association through groupings which must be located on their surfaces, the effect of various surface-active agents on the progress of the phenomenon was determined. Neutral detergents such as the Tweens did not affect either the rate of coacervation or the amount of product formed. This was not so, however, with cationic and anionic surface-active

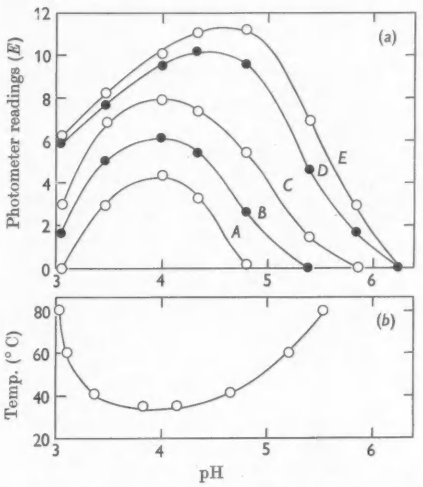


Fig. 1. (*a*) Variation of the extent of coacervation (measured as extinction) with the pH of solution at *I* 0·075. *A*, 28°; *B*, 35°; *C*, 41°; *D*, 60°; *E*, 80°. (*b*) A derived curve showing the variation of the temperature required for equal coacervation (arbitrarily chosen as 6 in Fig. 1*a*) with pH (cf. Partridge, Davis & Adair, 1955).

agents. The effect of sodium dodecyl sulphate and Cetavlon (cetyltrimethylammonium bromide) was determined at pH values both below and above the isoelectric point of the protein (Figs. 2*a*, *b* and 3*a*, *b*). At pH 3·4 the anionic sodium dodecyl sulphate causes an initial increase in coacervation followed by a pronounced drop as the concentration increases. At pH 5·4, however, the effect of this detergent is to inhibit coacervation steadily at all the concentrations tested (Figs. 2*a*, *b*). Cetavlon, having a positive charge, displays the opposite effect, inhibiting at pH 3·4 and activating at pH 8·4 (Figs. 3*a*, *b*). Similar results were obtained with other cationic and anionic detergents such as sodium dodecylbenzenesulphonate and cetylpyridinium bromide.

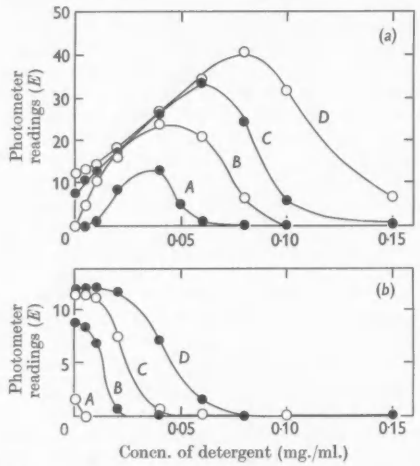


Fig. 2. (*a*) Effect of the anionic detergent sodium dodecyl sulphate on coacervation of α-elastin solution at pH 3·4 and *I* 0·15. *A*, 18°; *B*, 25°; *C*, 33°; *D*, 52°. (*b*) Effect of sodium dodecyl sulphate on coacervation at pH 5·4 and *I* 0·15. *A*, 41°; *B*, 57°; *C*, 69°; *D*, 85°.

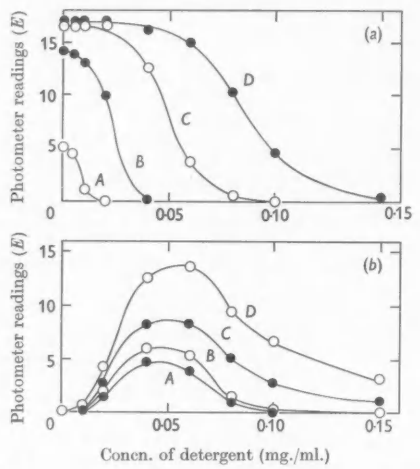


Fig. 3. (*a*) Effect of the cationic detergent Cetavlon on coacervation of α-elastin solution at pH 3·4 and *I* 0·15. *A*, 28°; *B*, 40°; *C*, 53°; *D*, 67°. (*b*) Effect of Cetavlon at pH 8·4 and *I* 0·15. *A*, 14°; *B*, 24°; *C*, 38°; *D*, 68°.

*Effect of elastase on coacervation*

Soluble elastin was dissolved in borate buffer, pH 8·45, mixed with elastase dissolved in the same buffer and incubated at 37°. Samples were withdrawn after various intervals and the enzyme was inactivated by heating for 4 min. at 100°. Part of each sample was adjusted to pH 4, diluted and heated to 40°. There was a steady release of amino groups, whereas the power to coacervate was lost much more rapidly. The addition of previously inactivated enzyme was without effect.

*Chemical and physical characterization of soluble elastin*

*Amino acid distribution.* No attempt was made to perform a quantitative analysis. The solid elastin and the preparations of α-elastin were hydrolysed with 5N-hydrochloric acid under the same conditions, and the resulting amino acid mixtures were subjected to two-dimensional paper chromatography. The qualitative amino acid spectrum of both types of protein is the same.

*Electrophoresis and isoelectric point.* When a crude product of elastolysis is subjected to paper electrophoresis at pH 8·6, the separation is poor. At pH 5·2 three fractions can be distinguished; the main component (α-elastin) migrates with the same speed as glucose. The best enzymic preparation of α-elastin moves as a well-defined band and its mobility is consistent with an isoelectric point of 5·0. This agrees with the isoelectric point obtained from the titration curve in 1 % sodium chloride solution (4·8).

*Ultraviolet spectrum.* The solution of α-elastin in borate buffer gives a smooth absorption curve with a rather flat but definite maximum at 275 mμ. The solution obeys Beer's law and the absorption coefficient at 275 mμ is 6·5. This low value is not unexpected in view of the small amount of tyrosine and the absence of tryptophan in both solid and soluble elastin.

*Viscosity and sedimentation.* An aqueous solution of α-elastin is not very viscous (intrinsic viscosity at 37° in borate buffer, pH 8·6, is 7·1 ml./g.). There is no increase in viscosity if the elastin solution is heated at pH 4·5 in the presence of sufficient sodium

Table 1. *Properties of preparations of α-elastin*

Values in column 4 are the percentages of sample precipitated from a 0·5% solution by 30% trichloroacetic acid.

| Sample or treatment | pH optimum for coacervation | Free amino group content (moles/$10^5$ g.) | Ppt. (%) |
|---|---|---|---|
| Treatment with $H_2SO_4$ | 4·2 | 32 | 94 |
| Treatment with NaOH | About 4·5 | — | — |
| AP | 9·2 | 37 | 87 |
| EP | 9·2 | 42 | 83 |
| AP treated with NaOH | 4·5 | — | — |
| Elastolysis (crude) | 4·5 | 65 | 65 |
| Treatment with pepsin | 5·5 | 29 | 98 |
| Elastolysis (controlled) | 5·2 | 30 | 98 |
| Deaminated | 2·0 | 2 | 93 |

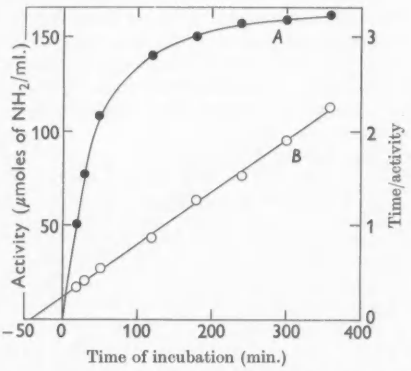


Fig. 4. A curve (*A*) relating the activity (expressed as moles of free amino groups/ml.) to time (●). The linear plot (*B*) of the ratio time/activity against time (○) shows that activity is a hyperbolic function of time. The substrate was elastin after $H_2SO_4$ treatment, 40 mg./ml., enzyme $E_2I$ (Hall & Czerkawski, 1959), 25 μg./ml., in borate buffer pH 8·5 and *I* 0·364 at 37°.

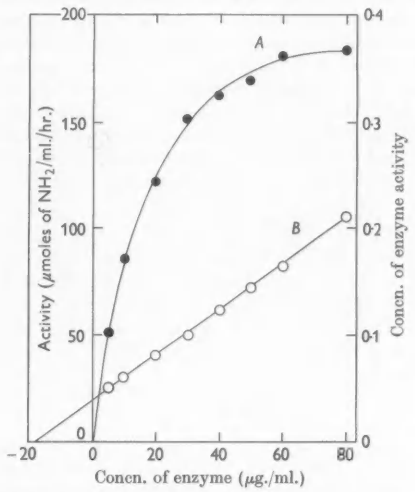


Fig. 5. A curve (*A*) relating the activity (expressed as the moles of free amino groups released in 1 hr.) to the initial concentration of elastase. The linear plot (*B*) of the ratio enzyme/activity against enzyme shows that this activity is a hyperbolic function of the enzyme concentration. The conditions are the same as in Fig. 4.

dodecyl sulphate to prevent coacervation. The implications of this observation are discussed below. Two samples of soluble elastin were subjected to sedimentation in a Spinco model E ultracentrifuge (1% solution in acetate buffer, pH 5·2). The α-elastin prepared with sulphuric acid gave a diffuse peak, which rapidly flattened and eventually disappeared. This material was obviously polydisperse. The soluble elastin prepared by the controlled action of elastase, however, gave a sharp symmetrical peak, indicating a high degree of homogeneity and confirming the electrophoresis results. The value of $S_{22,w}$ for these two proteins was 1·67 and 1·68 s respectively. A summary of the properties of various preparations is given in Table 1.

*Kinetics of the reaction between elastase and soluble elastin*

The initial rate of the reaction between elastase and soluble elastin is very fast (curve *A*, Fig. 4) and then falls off gradually, but does not proceed to completion within reasonable limits. Thus it is difficult to measure the initial reaction velocity and it is more convenient instead to measure the activity over a finite period. This is defined as the number of amino groups released in 1 hr. at 37° at the optimum pH. This optimum was 8·45 and was identical both with that for the elastolysis of the solid protein and with the isoelectric point of elastase. The activity is directly proportional to the starting concentration of substrate up to 30 mg. of α-elastin/ml. and varies hyperbolically with the enzyme concentration at the commencement of the reaction (curve *A*, Fig. 5). These relationships between elastin and elastase are equally applicable if the activity is measured in

terms of the material precipitable by trichloroacetic acid; this indicates that the proteolytic fission of the α-elastin molecule is stepwise rather than random, since precipitability by trichloroacetic acid is an approximate measure of particle size and, in random splitting, the decrease in molecular weight per unit of released amino group would increase rapidly.

A typical example is shown in Fig. 6, which relates the release of amino groups to the amount of non-precipitable material produced. Although there is good linearity, the straight line does not pass through the origin. The intercept on the ordinate is even greater with substrate prepared by pepsin digestion of solid elastin and is almost negligible for the material produced by attack with elastase in the presence of sodium dodecyl sulphate. The linear portions of the various curves are, however, approximately parallel.

Since the non-precipitable material produced is expressed in g./ml. and the amino group release is given in moles/ml., the slope of the line gives directly the approximate molecular weight of the unit which is split off in a stepwise fashion. The value is approximately 600, and is independent of the method of preparation within the limits of experimental error.

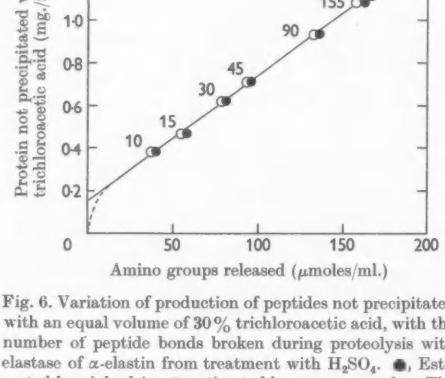


Fig. 6. Variation of production of peptides not precipitated with an equal volume of 30% trichloroacetic acid, with the number of peptide bonds broken during proteolysis with elastase of α-elastin from treatment with $H_2SO_4$. ●, Estimated by ninhydrin; ○, estimated by copper complex. The numbers on the curve refer to the time of elastase action.

Table 2. *Relative rates of digestion of various soluble elastin preparations*

Values are expressed in terms of the release of amino groups/$10^5$ g. of elastin protein/min.

| Substrate | Rate of release of amino groups (moles of $NH_2$/$10^5$ g. of elastin/min.) |
|---|---|
| α-Elastin from $H_2SO_4$ treatment | 1·0 |
| AP elastin | 0·4 |
| α-Elastin from elastolysis | 0·6 |
| Later stages of elastolysis | 0·3 |

The various detergents show marked differences in their ability to affect the rate of attack by elastase on α-elastin compared with their effect on elastolysis. The action of elastase on soluble elastin is only slightly affected by even high concentrations of Cetavlon, whereas the anionic detergents have almost no effect.

In Table 2 are shown the relative rates of digestion of various preparations. The slightly lower rate for the esterified fraction AP is probably due to the fact that at pH 8·45 and at a temperature of 37° most of this fraction is in the form of a coacervate and hence, in contradistinction to the other reactions, this particular one is heterogeneous and not homogeneous.

## DISCUSSION

### *Coacervation*

The pH optimum for coacervation is close to the isoelectric point of α-elastin and the extent of coacervation increases with temperature. Thus there are apparently two main requirements for coacervation, namely net neutrality of charge on the protein and a high degree of ionization. Other factors may, and in all probability do, contribute to the general mechanism, but for simplicity it may be assumed that coacervation is a reaction between positive and negative charges on adjacent molecules with the formation of salt linkages. The rapid disappearance of coacervation under the action of elastase may indicate that the phenomenon cannot take place when the particle size is below a critical value. On the other hand, the action of elastase may be to remove certain important polar groupings from the molecules. Below its isoelectric point the protein carries a net positive charge which will attract and combine with anionic detergents via their polar groupings and partially via hydrophobic acids. This combination will tend to make the protein molecule more neutral and will result in increased coacervation. The elastin molecule is, however, mainly non-polar and more of the detergent will be adsorbed through its non-polar hydrophobic regions by van der Waals forces than by salt linkages through its limited supply of polar groups. Consequently a net negative charge will be imparted to the molecule as a whole and there will be a progressive decrease in coacervation as the concentration of detergent increases.

At pH values below the isoelectric point of the protein, cationic detergents will combine solely by non-specific adsorption, thus continuously increas-

ing the net positive charge and steadily inhibiting coacervation. Above the isoelectric point, however, the protein carries a negative charge. It follows that the effects of the anionic and cationic reagents will be reversed: the former will show inhibition and the latter activation followed by inhibition as the concentration increases.

The phenomenon of coacervation is thus markedly dependent on the electric charge of the particles. Although the purely physical uncoiling and intertwining of particles proposed by Partridge *et al.* (1955) might be a contributing factor, the process is probably mainly due to a salt-like combination of charges on the protein.

The fact that there is no increase in viscosity under the conditions which would normally lead to coacervation indicates that it is not a purely physical process. However, the main argument against such a mechanism is the rapid reversibility of the process of coacervation (only a little shaking would intertwine a number of short helical springs and no amount of random movement would separate them again, unless influenced by a directive force).

Wood (1958) has prepared the so-called regenerated elastin by prolonged heating of the coacervate. The regenerated elastin is not soluble in water or dilute buffer, but it dissolves rapidly when acted on by elastase. This material may be the product of a purely physical and irreversible intertwining of α-elastin molecules.

### *Kinetics of homogeneous reaction*

Preliminary results of the study of the homogeneous reaction between elastase and α-elastin have been reported by Czerkawski (1958) and the present observations agree with those advanced earlier. The reaction can be represented accurately by an empirical relationship which is of the same form as equation (3) [see the straight lines (*B*), in Figs. 4 and 5].

The elastin prepared with sulphuric acid is probably homogeneous chemically, but there is no doubt that it is polydisperse with respect to the molecular size. At first sight it might be considered a poor choice of substrate for kinetic studies, but the fact that it is produced by a predominantly random fission of solid elastin turns out to be advantageous. It means that the resulting material is chemically very similar to the solid elastin and that, at least in theory, any kinetic results obtained from its reaction with elastase may be extrapolated to the more complex solubilization of elastin. In this respect the soluble protein produced by the action of elastase is inferior, although it is quite monodisperse.

The kinetic studies of the homogeneous reaction are helpful in the theoretical derivation of an equation which is very similar to the empirical one. The fact that the reaction is first-order with respect to the substrate indicates the fast formation of an enzyme–substrate complex, and the fact that it remains of this order throughout implies a stepwise degradation of the substrate.

The reaction appears to consist of two processes, one fast, one slow. The former may be identified with the fission of specific bonds and may lead to the solubilization of solid elastin (bonds which have survived the indiscriminate acid hydrolysis), and the latter appears to be the stepwise degradation of the soluble material.

If it is assumed that the enzyme is adsorbed instantaneously on elastin according to the Langmuir isotherm, then the fraction of sites occupied by the enzyme, $\theta$, is $E_0/(K+E_0)$, where $E_0$ is the initial concentration of the enzyme. If it is also assumed that the substrate contains $n_1$ bonds which are attacked rapidly and $n_2$ that are attacked slowly, and that the latter predominate, then if $x$ is the number of bonds split in time $t$, the reaction velocity is given by

$$dx/dt = k_1(n_1\theta - x) + k_2 n_2 \theta, \qquad (1)$$

where $k_1$ and $k_2$ are the velocity constants for the fast and slow reactions respectively.

The above equation can be integrated and if the time interval used is not too large it can be simplified, giving finally

$$x = \left(R + \frac{k_1}{k_1}\right) n_2 \frac{E_0}{K+E_0} \frac{t}{1/k_1 + t} \qquad (2)$$

where $R = n_1/n_2$.

All the constants in the above equation can be obtained experimentally. If $t/x$ is plotted against $t$ at constant enzyme concentration the intercept on the $t$ axis gives $1/k_1$, and is expressed in reciprocal time units. Similarly a plot of $E_0/x$ against $E_0$ gives $K$ which is measured in the same units as the enzyme concentration. If the smallest degraded elastin unit has a molecular weight of 600, the solution containing $a$ mg. of soluble elastin/ml. must contain $1{\cdot}67\,a$ μmoles of such units/ml., this may be identified with $n_2$. The value of $n_1$ may be estimated from the steep portion of the activity–time curve, and so one may determine $R$. Thus all the quantities except $k_2$ are known and therefore this may also be calculated. However, if one puts $n_1 = 0$ (for the substrate prepared by controlled elastolysis), one obtains:

$$x = n_2 \frac{E_0}{K+E_0} \frac{t}{1/k_2 + t} \qquad (3)$$

and thus $k_2$ may also be obtained directly.

The derived equation contains a number of constants which have a definite physical meaning and to which one can assign the appropriate dimensions.

The effect of detergents on the homogeneous reaction is interesting practically. The elimination of the secondary reaction by sodium dodecyl sulphate can be made use of in the preparation of an α-elastin which is far more uniform than that obtained by any of the more drastic chemical methods.

## SUMMARY

1. Several methods of preparation of soluble elastin are described and it is shown that the best material is obtained by controlled elastolysis.

2. Coacervation is taken as best characterization of α-elastin and the most suitable conditions for the measurement of this phenomenon are investigated. The effect of detergents and elastase on this phenomenon are also studied.

3. It is concluded that the process of coacervation of α-elastin is complex and is mainly brought about by salt-like interaction between charged centres of the protein molecules.

4. The kinetic studies of the reaction between α-elastin and elastase give rise to an empirical equation. It is shown that only a few reasonable assumptions and the observed facts lead to a theoretical equation which is of the same form as the empirical one. The constants of the equation have a sound physical meaning and can be obtained experimentally.

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*Biochem. J.* (1961) **80**, 128

# The Reaction between Elastase and Elastic Tissue

## 5. GROUPINGS ESSENTIAL FOR ELASTOLYSIS

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(*Received* 3 *October* 1960)

The amino acid distribution of elastin has been determined by numerous workers (for review see Hall, 1959), and the values obtained show that the molecule is built up from predominantly non-polar amino acids. Thus $10^5$ g. of the protein contain only 28 moles of dibasic acids, 12 moles of diamino acids, 33 moles of aliphatic hydroxy acids and 7 moles of tyrosine (calculated from the figures reported by Partridge & Davis, 1955). The remainder (1120 moles) consists of various non-polar amino acids. Of the former group the amino acids containing the weakly polar aliphatic hydroxyl groups predominate. This rather unusual distribution of amino acids confers a considerable degree of chemical stability on the protein, thus making it admirably suited to chemical studies. Elastin is dissolved by the proteolytic component of elastase (Hall & Czerkawski, 1959) and to a far smaller extent by certain other proteolytic enzymes of plant origin (Thomas & Partridge, 1960). This process, commonly known as elastolysis and which we wish to distinguish from the subsequent degradation in solution (Hall & Czerkawski, 1961), is a striking example of a reaction in which a solid substrate is made to pass into solution.

Banga & Schuler (1953) were unable to identify any non-protein amino groups in the products of the reaction between elastase and elastin. They interpreted this as indicating that the solubilization of elastin was accomplished without the fission of

peptide linkages. They suggested that a fibrous form of elastin was converted into a globular form merely by the fission of hydrogen bonds. Partridge & Davis (1955) showed that the complete solubilization of elastin was accompanied by the release of a polydisperse material that had a very high amino group content [(121 moles/$10^5$ g. of protein as compared with 17 moles deduced from titration curves for native elastin by Bendall (1955)]. The production of this material was, however, only accomplished by incubation with the enzyme for a period which was three times as long as was necessary for the dissolution of the solid. Thus it is not strictly true to say that these terminal amino groups were liberated during elastolysis, since many of them may have been formed by the fission of peptide linkages after the elastin had passed into solution. In view of the preliminary results obtained for the kinetic analysis of the reaction between elastase and the soluble elastin (Czerkawski, 1958) and the differences that could be observed between the rates of amino acid release during the two stages of the reaction, it was decided to investigate the chemistry of these two component stages of the overall reaction.

## MATERIALS AND METHODS

*Standard enzyme and substrate.* The enzyme employed throughout these experiments was the very active preparation $E_2I$, obtained by the method described by Hall & Czerkawski (1959). The preparation employed had an activity of 370 units/mg. (cf. definition of activity in the paper quoted above) and was used at a concentration of 5–10 $\mu$g./ml. in a system containing 50 mg. of substrate suspended in 10 ml. of borate buffer ($I$ 0·364, pH 8·45).

The standard substrate preparation was from ox ligamentum nuchae, the powder (40–60 meshes/in.) being freed of collagen by the method described by Hall (1955).

*Estimation of elastolytic activity.* Elastolysis was carried out under the conditions of Hall & Czerkawski (1959), and the amount of protein passing into solution measured by means of the biuret reaction. If pretreatment of the substrate resulted in the formation of a coloured product which gave a coloured solution after elastolysis, the degree of solubilization was determined by the interferometric method of Hall & Czerkawski (1959).

*Proteolytic activity measurements.* Proteolysis as opposed to solubilization was measured by the methods described by Hall & Czerkawski (1961), as also were assessments of molecular size based on the ability to form coacervates and precipitation with trichloroacetic acid.

*Determination of methoxy and acetyl groups.* The extent of esterification of certain modified substrates was measured by means of the Zeisel method adapted by Grant (1945). Acetylation of the substrate was determined by the method of Herriot & Northrop (1934), in which acetic acid is displaced by citric acid and distilled at a pressure of 20 mm. Hg. There are no complications due to atmospheric $CO_2$.

## RESULTS

### *Proteolytic activity during elastolysis*

The amount of protein released and the number of terminal amino groups liberated during the progress of the elastolytic reaction are shown in Fig. 1*a*. The number of amino groups decreases, passes through a minimum and then increases again towards the end of the reaction when all the solid has been taken into solution. The lowest value reached is about 70 moles/$10^5$ g. of protein as compared with $\alpha$-elastin which has about 30. The percentage of trichloroacetic acid-precipitable material (Fig. 1*b*) and the ability to form coacervates (Fig. 2) pass through maxima which are coincident with the minimum in Fig. 1*a*.

### *Elastolysis in the presence of sodium dodecyl sulphate*

Sodium dodecyl sulphate was added to a standard system containing 50 mg. of elastin in 8 ml. of borate buffer, pH 8·45, so that its final concentration in 10 ml. was 2 mM, and the mixture incubated

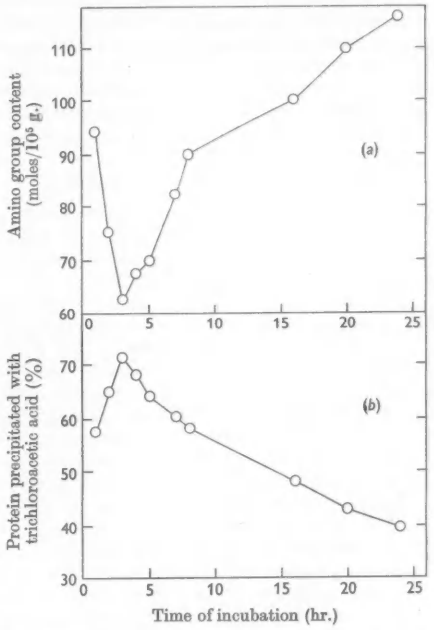


Fig. 1. (*a*) The amino group content (measured by the ninhydrin reaction) during various stages of elastolysis under normal conditions. (*b*) The extent of precipitation by trichloroacetic acid (equal volume of 30% trichloroacetic acid) of the protein released during elastolysis.

at 37° for 30 min. Before the addition of the enzyme (in a further 2 ml. of buffer) the system was chilled to 4° and this temperature was maintained for 10 min. after the enzyme had been added. This enabled the enzyme to react with the substrate without suffering denaturation by the detergent. Subsequent incubation was carried out at 37° for various periods. In controls the detergent was replaced by 2 mM-NaCl. Fig. 3*a* shows that under these conditions the average α-amino group content of the liberated protein remains approximately constant at the low value of 30 moles/$10^5$ g., and similarly the material passing into solution is almost completely precipitated by trichloroacetic acid at any stage of the reaction (Fig. 3*b*). It should be noted that sodium dodecyl sulphate is a powerful activator of elastolysis (Table 1).

### *Ability to coacervate during the course of elastolysis*

The pH of the filtrates from elastolysates obtained after various periods of incubation was adjusted to 4·0; the solutions were diluted suitably and warmed to 40°. The degree of coacervation per unit mass of protein in solution was maximum during the middle third of the reaction. It was difficult to measure the extent of coacervation in the presence of sodium dodecyl sulphate, for this reagent suppresses this process at pH 4 and increases it at pH 3. After exhaustive extraction of the detergent, however, the material obtained by elastolysis in its presence demonstrated coacervation in the normal way at pH 4·0.

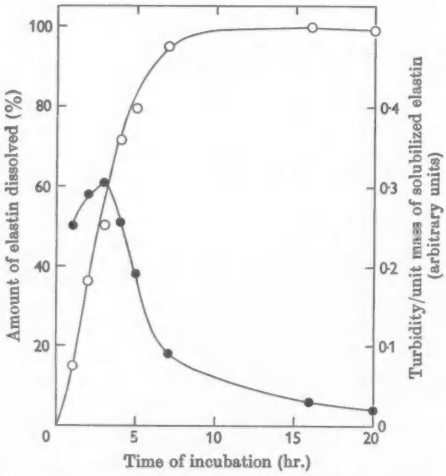


Fig. 2. The solubilization–time curve (○) for the system containing 50 mg. of elastin in 10 ml. of borate buffer, pH 8·5, *I* 0·364, and 8 μg. of elastase/ml. The power to form a coacervate is expressed as the ratio: turbidity/protein concn. (●).

### *Function of carboxyl groups in elastolysis*

Preliminary observations showed that the cationic detergent Cetavlon acts as a powerful inhibitor of elastase (0·1 mM-Cetavlon produces 50% inhibition). This proved to be a general property of cationic detergents and appeared to be due to the stable binding of the detergent molecule to the substrate. There were two possibilities: either the detergent was bound by its long non-polar tail to the non-polar regions of elastin, or it was bound through its positively charged head to the relatively few negatively charged centres. The results obtained with neutral and acidic detergents indicated that probably the latter was correct; thus the effect of other methods of blocking the negatively charged

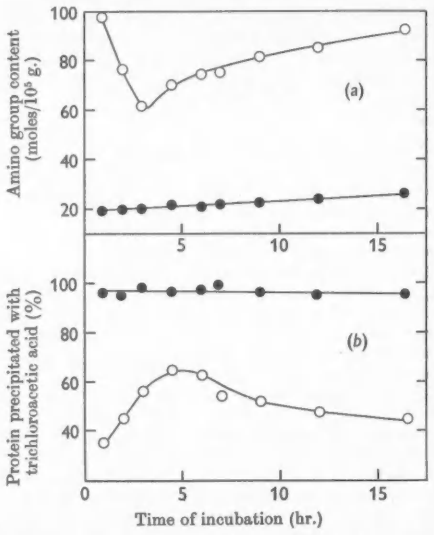


Fig. 3. Amino group content (*a*) and extent of trichloroacetic acid precipitation (*b*) of material released during various stages of elastolysis in the presence of sodium dodecyl sulphate (●) and controls in the presence of NaCl of the same ionic strength (○).

Table 1. *Effect of detergents on the action of elastase on the solid and soluble elastins*

| Detergent (0·5 mM) | Change in activity | |
|---|---|---|
| | Homogeneous | Heterogeneous |
| Sodium dodecyl sulphate | 4% activation | 105% activation |
| Cetavlon | 9% inhibition | 81% inhibition |

groups in elastin on the progress of elastolysis was studied.

*Esterification.* Elastin powder (2·5 g.) was suspended in 100 ml. of methanol containing 0·1 N-HCl and the mixture kept at room temperature with intermittent stirring for 3 days. It was then filtered and washed alternately with many changes of water and acetone. The solid was finally washed with acetone and dried at 37°. Elastin thus esterified was quite resistant to elastase, being only one-fifth as susceptible as the standard substrate (Table 2). The ester could be saponified with 0·01 N-NaOH at 37°, and yet quantitative estimations of methoxy groups showed that esterification of 14 of the possible 28 sites had occurred (Fig. 4*a*). However, original activity could be regenerated in only a few minutes, and further mild hydrolysis gives a vastly increased susceptibility to elastase (Fig. 4*b*).

Table 2. *Effect of various treatments of elastin on its susceptibility to elastase*

| Pretreatment of substrate | Change in susceptibility (%) Increase | Decrease | % of enzyme adsorbed* |
|---|---|---|---|
| Standard elastin | 0 | 0 | 25 |
| Esterification | 0 | 80 | 0 |
| *O*-Acetylation | 0 | 50 | 59 |
| Thiohydantoin | 0 | 20 | — |
| Full acetylation | 0 | 20 | — |
| Dinitrophenylation | 0 | 30 | — |
| *N*-Acetylation | 50 | 0 | 37 |
| Deamination | 50 | 0 | — |
| Glyoxylation | 190 | 0 | 80 |
| Iodination | 0 | 0 | — |

* Dashes indicate that adsorption experiments were not performed on these substrates.

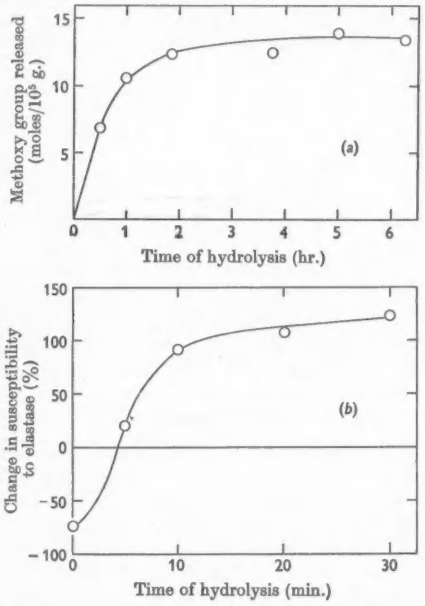


Fig. 4. Saponification of esterified elastin. (*a*) Quantitative release of methoxy groups during hydrolysis with 0·01 N-NaOH at 37°. (*b*) Susceptibility of esterified elastin to elastase. (Both the test samples and standard elastin powder were treated with 0·01 N-NaOH for the indicated time interval.)

To decide whether the carboxyl groups were terminal or those of amino acid side chains, a thiohydantoin derivative was prepared. The conditions under which this was attempted (30 min. at 100°) were considerably more drastic than those employed for the introduction of methoxy groups. Elastin (1·0 g.) was suspended in 40 ml. of a mixture of acetic acid and acetic anhydride (8:1) containing 500 mg. of $NH_4CNS$, and heated for 30 min. at 100°. After chilling and washing, the protein was dried with acetone. The product was 20 % less susceptible to elastase than was the starting material (Table 1). The whole of this loss of susceptibility could, however, be attributed to acetylation, since a similar experiment under identical conditions but in the absence of the $NH_4CNS$ afforded a modified substrate that also had 20 % less susceptibility to elastase than had native elastin.

### *Function of amino and hydroxyl groups*

Acetylation under the conditions of the control experiment for the thiohydantoin reaction, although indicating that acetylation affects certain groupings that are essential for the elastase–elastin reaction, was too drastic a method since although saponification removed the acetyl groups, the susceptibility of the regenerated substrate was considerably greater than that of the starting protein. From this it would appear that during the acetylation process degradation may occur.

Milder conditions of acetylation can be used to acetylate particular reactive groups and two such methods have been employed. Elastin (2·0 g.) was suspended in 15 ml. of citrate buffer, pH 4·0, and 5 ml. of acetic anhydride added in small portions at 5° throughout a period of 5 hr. The mixture was kept at 5° for 18 hr., filtered, washed and dried as before. The other method, which is claimed to acetylate only hydroxyl groups (Bello & Vinograd, 1956), also utilizes acetic anhydride at low temperatures, but the reaction is allowed to proceed in the presence of small amounts of $HClO_4$.

Quantitative estimation showed that 56 acetyl groups had been introduced/$10^5$ g. of fully acetyl-

ated elastin. Saponification of these groups indicates that they are not all identical. About 40 can be saponified easily, whereas the remaining 16 are only removed with difficulty (Fig. 5). *O*-Acetylation reduces the activity by about 50 % (Table 1) and it is significant that a 2 hr. treatment with 0·1 N-NaOH at 37° results in complete regeneration of the elastase susceptibility of this substrate. Acetylation under milder conditions blocks all the amino groups and about 13 hydroxyl groups, resulting in a 50 % increase in elastolysis.

*Deamination.* Elastin (1·0 g.) was suspended in 15 ml. of citrate buffer, pH 5·0, and chilled to 5°. The suspension was mixed with 50 ml. of water containing 1·0 g. of $NaNO_2$ and kept at a temperature of between 2° and 5° for 18 hr. When filtered off, washed and dried, the product was a yellow powder. Treatment of this substrate with elastase resulted in increased elastolysis (Table 1), an observation which was in agreement with the results of *N*-acetylation. Although the amount of elastin protein passing into solution from the deaminated substrate was increased, the number of amino groups released during the initial stages of the same reaction decreased.

*Iodination.* The introduction of $I_2$ into elastin, under conditions favouring substitution in the tyrosine residue (pH 9·0, 0·01 M-$I_2$ in 0·5 M-KI at room temperature for 40 min.), has no effect on the rate of elastolysis, although the amount of $I_2$ consumed (15 moles/$10^5$ g. of protein) is consistent with disubstitution of the seven tyrosine molecules of elastin.

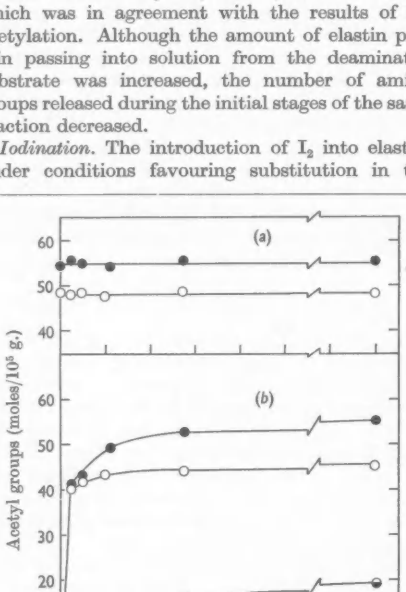


Fig. 5. Release of acetyl groups from acetylated elastin during mild hydrolysis with 0·05 N-NaOH at 37°. (*a*) The sum of acetyl groups held and released during hydrolysis of fully acetylated (●) and *O*-acetylated (○) elastin. (*b*) The amount of acetyl group released during saponification of fully acetylated (●), *O*-acetylated (○) and mildly *N*-acetylated (◒) elastin.

*Dinitrophenylation.* Elastin was treated with 1-fluoro-2:4-dinitrobenzene according to Sanger (1945). The susceptibility of this yellow material to elastase was decreased by 30 %. The amount of dissolved protein was measured in the interferometer, because the coloured protein interfered with the biuret reaction.

*Introduction of new carboxyl groups.* Elastin was treated with glyoxylate at pH 9·3 and with monobromoacetate at pH 10 for 4 hr. as recommended for collagen by Sykes (1952). Both these procedures, which introduce new carboxyl groups as well as blocking amino groups, increase the susceptibility of elastin to elastase (Table 1).

### *Adsorption of elastase on elastin*

Hall & Czerkawski (1959) showed that elastase is rapidly adsorbed on elastin at 4° at the optimum pH of the system without any appreciable elastolysis occurring. This phenomenon has been used to assess how modification of the substrate affects its ability to pick up elastase.

Elastin (50 mg.), esterified elastin and *O*-acetylated elastin were suspended in 2 ml. of borate buffer, pH 8·45, and chilled to 4°. The enzyme solution was then added to bring the volume to 5 ml. and the mixture kept at 4° for 5 min. The solid was removed by centrifuging at the same temperature. A sample of the supernatant was added to standard elastin suspended in fresh buffer, and incubated at 37° for the standard period of time. The difference between the degree of elastolysis of a control and the test sample was a measure of the amount of enzyme adsorbed on the substrate. Typical results are presented in Table 1 (third column).

## DISCUSSION

The empirical equation which was derived to explain the reaction between elastase and elastin (Hall & Czerkawski, 1961) consists of three terms: a linear function of substrate concentration, a hyperbolic function of enzyme concentration and a function of time. Differentiation of this equation with respect to time gives an equation representative of the rate of the reaction and directly proportional to the amount of substrate available, to the same hyperbolic function of enzyme concentration, and again to a function of time. The amount of solubilized elastin increases rapidly owing to the sigmoid shape of the elastolysis–time curve. Part of

the enzyme may be assumed to become adsorbed on the surface of the solid elastin, part will diffuse more slowly into the solid and part will remain in solution. Thus the solution becomes progressively poorer in enzyme and richer in soluble elastin. This latter material will also be able to pick up elastase from solution and the direct outcome will be a relatively small increase in the rate of the secondary reaction between elastase and the soluble product of the first reaction. Consequently the solubilized protein becomes progressively richer in the unattacked soluble material in agreement with the experimental results given in Fig. 1.

In the later stages of elastolysis the substrate for the secondary reaction begins to predominate, and the process of diffusion, which tends to diminish the amount of enzyme available in the solution, not only stops but is reversed as the solid within which it is trapped passes into solution. At the same time as the amount of substrate for the first reaction decreases the rate of solubilization decreases until the rate of the second reaction eventually surpasses it. When all the elastin is dissolved the second reaction slows down but does not stop. The number of terminal amino groups approaches the value of 120 moles/$10^5$ g. of protein, in agreement with the results of Partridge & Davis (1955). The terminal amino acid content of solid elastin is negligible (0·3 mole/$10^5$ g. according to Partridge) and when the secondary reaction is inhibited by detergent the product of elastolysis contains about 30 such groups. It is obvious that if both reactions are brought about by the same enzyme, as would appear to be the case in view of the purity of the elastase preparation (Hall & Czerkawski, 1959), the respective reactions must be different.

The reaction with the solid substrate appears to require the initial adsorption of the enzyme on the substrate followed by their interaction. The results of the adsorption experiments indicate that the presence of a free carboxyl group is essential for this first half of the reaction. It is therefore not surprising that esterification has such a marked effect on elastolysis, since if the enzyme and its substrate are not able to combine no reaction can take place in such a heterogeneous and consequently highly localized system. The nature of the carboxyl groups involved in this adsorption reaction can be deduced from the failure of thiohydantoin reaction. Such cyclizations take place only with α-carboxyl groups and hence the ones which are available in elastin must be the side-chain carboxyl groups of glutamic acid and aspartic acid. Although the carboxyl group appears to be all-important in attracting the enzyme to the substrate, the presence of an unsubstituted hydroxyl group also seems to be necessary for the actual reaction to proceed. It need not necessarily be inferred that the carboxyl and hydroxyl groups are present on adjacent amino acid residues nor even that they are on the same peptide chain. They may merely be brought into close proximity by the spatial convolutions of the elastin molecule. The net result must be, however, the production of a hydroxyl–carboxyl complex capable of reacting with a single centre on the enzyme and not two separate groupings reacting with widely separate centres, since the incorporation of glyoxylate into the molecule, which introduces fresh hydroxyl and carboxyl groups adjacent to one another, results in a marked increase in activity. But monobromoacetic acid introduces no hydroxyl group and yet gives an increase.

Both the hydroxyl and the carboxyl groups must therefore react with a single relatively small centre in the enzyme. Hall (1954) suggested that the enzyme and the substrate might be united through a calcium atom and that the calcium atom was held by chelate bonds to both proteins. The essential nature of carboxyl and hydroxyl groups which could afford such a chelating centre on the substrate is very favourable to this hypothesis.

Elastolysis results not only in the dissolution of elastin but also in its degradation whilst in solution. Table 1 indicates that although the enzyme appears to be unitary in nature it has quite a different mode of action on solid and soluble elastin. Carboxyl and hydroxyl groups are essential for the reaction with native elastin. The presence of free amino groups appears to have an adverse effect on elastolysis, but does not affect the secondary reaction. Involvement of amino groups may explain the anomalous results of the adsorption experiments with *O*-acetylated substrate. Partial acetylation of the amino groups could result in a charge redistribution and an enhanced adsorption on the carboxyl group.

The reaction between elastase and elastin is therefore a complex system with at least two parts, which are to a certain extent in competition. The first reaction consists in adsorption of elastase on solid elastin through the side-chain carboxyl groups of glutamic acid and aspartic acids and the possible formation of a chelated link through calcium ions and the hydroxyl groups of tyrosine and possibly other residues bearing such groups. As the reaction proceeds and soluble elastin appears in solution, an increasing proportion of elastase becomes bound to this second substrate. This partition of the enzyme between the two substrates can account for some of the changes occurring during the course of the overall reaction. The adsorption of elastase is increased by sodium dodecyl sulphate, which imparts a net negative charge to the protein. This results in an increased elastolysis (appearance of protein in solution) at the expense of proteolysis (appearance of amino groups). This differentiation between elastolysis and proteolysis is more apparent than real

since the appearance of 30 amino groups in elastin indicates a limited proteolytic reaction in the first stage.

When esterified elastin is subjected to very mild alkaline hydrolysis its susceptibility to elastase reverts to normal long before all the carboxyl groups are unmasked. Moreover, when all the methoxy groups are removed, the activity is much greater than that of the standard powder treated with alkali under identical conditions. It is possible that elastin contains some linkages (probably ester) which are particularly susceptible to hydrolysis by ethanol. Such linkages may be purely internal or might be utilized in binding traces of other substance, such as lipid or polysaccharide. The enzymic cleavage of such strategic bonds might constitute an additional third reaction.

## SUMMARY

1. The proteolytic activity of elastase during solubilization of elastin is investigated by measuring the release of free amino groups and the magnitude of the average particle size. It is shown that the secondary process may be largely eliminated by elastolysis in the presence of an anionic detergent.

2. Chemical modification of certain side-chain groups of elastin shows that the side-chain carboxyl and the tyrosine hydroxyl groups are necessary for elastolysis. The former are directly concerned in adsorption of elastase on elastin. The amino groups are not directly concerned in elastolysis, but their blockage or removal gives an increase in activity probably due to the increased electronegativity of elastin.

3. The results of chemical studies are discussed in the light of the postulated two reactions.

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*Biochem. J.* (1961) **80**, 134

# The Reaction between Elastase and Elastic Tissue

## 6. THE MECHANISM OF ELASTOLYSIS

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(*Received* 3 *October* 1960)

Some hypotheses on the mechanism of elastolysis have been discussed by Hall & Czerkawski (1959, 1961*a*, *b*). New information has been given about the two postulated reactions, namely solubilization and secondary degradation in solution. The present paper introduces data in support of a postulated preliminary reaction and correlates it with the other two.

The reaction between solid elastin and elastase is preceded by the adsorption of the enzyme on the substrate through the side-chain carboxyl groups. Blockage of such groups prevents adsorption and, therefore, reaction, whereas an increase in their number increases both these considerably. However, such adsorption is normally confined to the surface or the layers close to the surface of elastin particles (Czerkawski, 1960), and solubilization would be very inefficient unless there were a loosening, permitting the ensuing permeation of elastin by elastase. Such a process, although important, would be difficult to detect chemically; but, it would result in structural changes, which are bound to produce large changes in the elastic properties of elastin. The elasticity of this material has previously

been measured only before and after enzymic attack (Wood, 1954). Here such measurements have been attempted during elastolysis.

## METHODS AND MATERIALS

*Elastin.* Fresh ox ligament was cut into longitudinal sections 500 $\mu$ thick and about 8 cm. long on a freezing microtome. These sections were then extracted repeatedly at 100° with aq. 1% acetic acid in a Soxhlet apparatus to remove most of the collagen. The sections were spread on polythene sheets, frozen and cut with scissors into strips measuring approximately 1 cm. by 8 cm.

*Enzyme.* The elastase used was purified as described by Hall & Czerkawski (1959).

*Buffer.* The load–extension curves were determined in borate buffer (pH 8·5, $I$ 0·364), since preliminary experiments had shown that the Young's modulus of elastin remained constant in the pH range 6–10.

*Load–extension apparatus.* This consisted of a jacketed vertical tube fitted with an adapter, which was provided with a stopcock and a glass hook at its lower end. A strip of elastin was suspended in acetone, and, when nearly dry, its ends were folded over and cemented into two small glass triangles. When quite dry the ends were painted with the same cement, to make them impermeable to water, and the complete systems were finally suspended for 16 hr. in borate buffer. The lower glass triangle was attached to the upward-pointing glass hook and the upper one was connected to a fine silk thread, which passed out of the top of the apparatus over a light pulley and was secured at the end to a light pan for weights. The extension was measured by means of a travelling microscope focused on the top of the strip. The strip was completely immersed in the buffer solution, which could be easily withdrawn through the tap at the bottom for analysis (ultraviolet absorption at 275 m$\mu$) and subsequently replaced in the vessel.

The temperature was kept constant by passing water from an external thermostat through the outer jacket.

## RESULTS

*Extension at constant load.* The strip was suspended in borate buffer at 37° under a small constant load of 2 g. The enzyme was then added. The amount of elastin passing into solution was measured (extinction at 275 m$\mu$) and also the extension. The rate of solubilization had a low but constant value (3·6 %/hr.), and there was initially no change in extension. However, the latter began to increase rapidly, eventually becoming very great (about tenfold increase), resulting finally in breakage. The break occurred in the central portion of the strip and no previous irregularities of the strip could be detected visually. The time interval elapsing before break varied with the enzyme concentration.

*Effect of elastase on the Young's modulus of elastin.* The above results suggested that elastase produces a marked decrease in the Young's modulus ($E$) of elastin, and subsequently this was demonstrated directly. The strip was suspended in borate buffer in the same apparatus as described above, but the maximum extension was limited purposely by permitting the weighted pan to travel a distance of only 0·7 cm. The measurements for the load–extension curve, which was linear up to about 30 % extension, took 2–4 min. and the measurement of the extent of solubilization took about 1 min. The experiment was carried out at 37° in elastase solution containing 10 $\mu$g. of the enzyme/ml. The load–extension curves remained linear throughout and $E$ could be calculated from the slope and from the dimensions of the strip. Two corrections were applied to each of the values, both relating to changes in cross-sectional area, due to a small amount of plastic creep and to solubilization. The corrections were only of considerable proportions after prolonged elastolysis; for instance, when $E$ has decreased by 80 % these amounted to only 4 and 10 % respectively. The results are shown in Fig. 1.

## DISCUSSION

*Preliminary reaction.* It can be shown thermodynamically (Alfrey & Gurnee, 1956) that a decrease in the $E$ at constant load is accompanied by an increase in entropy and consequently in the amount of disorder. This could be brought about by the severance of certain strategic lateral bonds and

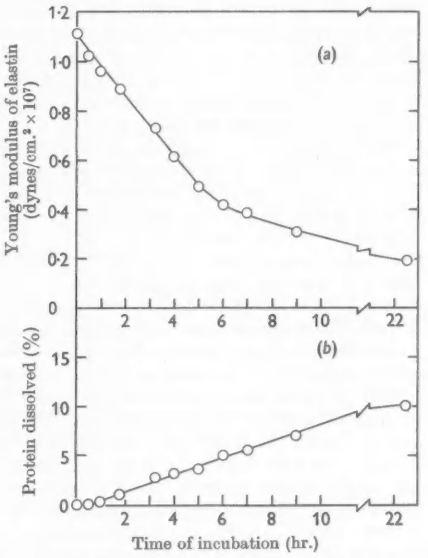


Fig. 1. (*a*) Change in Young's modulus of elastin during the early stages of elastolysis; (*b*) amount of protein released into solution.

the lengthening of the loosened longitudinal peptide chains. Treolar (1958) gives a formula which relates the molecular weight of a free chain fixed at both ends to the modulus of rigidity of the elastic substance of which it is composed, namely $n = RT\rho/M$, where $n$ is the modulus of rigidity, $\rho$ is density and $M$ is the molecular weight of such a chain. Now $n$ is related to $E$ and the Poisson's ratio $\rho$ by the well-known relation $n = E/2\,(1+\sigma)$. For elastin, the necessary calculations give a value for $M$ of 2600. The significance of this value is that it is doubled when only a very small fraction of the elastin has been dissolved under the action of elastase. Thus the early stages of elastolysis appear to be associated with the severance of lateral bonds together with an increased looseness of structure (cf. Banga & Balo, 1960).

*Mechanism of elastolysis.* The complete elastolytic reaction may therefore be at present pictured as follows. The enzyme is adsorbed on the available side-chain carboxyl groups of elastin (Hall & Czerkawski, 1961*b*). This may result in reaction or it may not. If there is no reaction the enzyme is simply attached to the substrate. On the other hand, another part of the enzyme (active centre) may come into contact with a specific site of the substrate and this may result in the fission of bonds and loosening of structure, but not necessarily in solubilization. This should be called the preliminary reaction and it would correspond to the slow phase of the elastolysis–time curve (Hall & Czerkawski, 1959). After a time a sufficient number of bonds will have been broken for particles to start passing into solution. The difference between preliminary reaction and solubilization might be only one of degree or it might involve quite different bonds. Nevertheless, an increase in the extent of the preliminary reaction must result in an increase of the rate of solubilization. The latter process results in production of α-elastin molecules, which then combine with some of the enzyme in solution.

The highly localized solubilization may also be speeded up by preferential adsorption on the carboxyl groups of the substrate, since reaction would be very slow if the process were purely random. The α-elastin is subject to a relatively slow stepwise degradation and therefore this material accumulates partly in solution and partly within the undissolved solid. This product eventually becomes inhibitory. However, the diminution in the rate of solubilization is caused mainly by a decrease in the availability of the solid.

*Elastolysis at macroscopic level.* Czerkawski (1960) has shown that relatively intact elastin is attacked preferentially from the ends of the fibres and that the rate of reaction depends on the exposed surface. It has long been known that the fresh tissue is attacked only very slowly by elastase. This agrees with the fact that the rate of metabolism of elastin in the tissues is very low (Slack, 1954). However, like any other tissue it must metabolize and there must be some process by which solubilization can be brought about. Moreover, arteriosclerosis leads eventually to lesions and it is difficult to visualize such a tough substance as elastin breaking down mechanically without some previous degradation brought about by a catalytic agent.

It is uncertain what causes the sclerotic lesion in the aorta. It may be that the tissue is weakened mechanically by deposition of fat or by calcification. The perpetually fluctuating stresses may result eventually in a small mechanical damage. Such a break could act as a trigger and open the way for elastolytic attack that will primarily produce a marked lowering of the elastic modulus of the affected part, and thus result in distension and eventually in dissection of the vascular wall.

A similar process, scaled down suitably, may take place in healthy tissues. Under such conditions all three reactions (the primary loosening, solubilization and stepwise degradation of soluble elastin) would proceed slowly, subject to many controlling factors, such as auxiliary enzymes or serum inhibitors. In spite of its slowness, the whole process is economic and efficient. The stepwise requirement prevents complete degradation and appears to leave units for possible resynthesis.

## SUMMARY

1. Many observations on the reaction between elastase and elastic tissue indicate that, in addition to solubilization and secondary degradation in solution, there is a third reaction that may be termed preliminary. Its presence may be demonstrated by a marked change in elasticity of elastin during early stages of elastolysis.

2. The preliminary reaction is correlated with the other two, and a possible complete mechanism of elastolysis *in vitro* and at macroscopic level is discussed.

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# Experiments on the Biosynthesis of Thiamine

## ISOTOPE-COMPETITION EXPERIMENTS WITH [$^{35}$S]SULPHATE IN CULTURES OF *SACCHAROMYCES CEREVISIAE*

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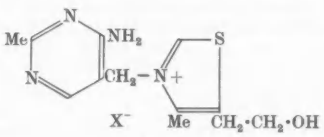
Although the structure of thiamine (I) was established and its chemical synthesis accomplished twenty-five years ago (Williams & Cline, 1936; Grewe, 1936; Andersag & Westphal, 1937; Todd & Bergel, 1937), little precise knowledge of its biosynthesis has become available. Many systems have been studied which have the ability to link together the pyrimidine (II; X = OH,OEt,$NH_2$, Br) and thiazole (III) fragments, but the routes by which these moieties are themselves biosynthesized are quite unknown.

The presence of the thiazole-ring system has recently been demonstrated in antibiotics, such as micrococcin P (Brookes, Fuller & Walker, 1957; Brookes, Clark, Fuller, Mijović & Walker, 1960), bottromycin (Waisvisz, van der Hoeven & te Nijenhuis, 1957) and thiostrepton (Kenner, Sheppard & Stehr, 1960; Bodanszky, Sheehan, Fried, Williams & Birkhimer, 1960), and the chemically generic relationship of the structures there encountered with those found in bacitracin F (Hausmann, Weisiger & Craig, 1955; Weisiger, Hausmann & Craig, 1955) and in bacitracin A (Newton & Abraham, 1953; Craig, Hausmann & Weisiger, 1954) indicates clearly that these structures are derived from cysteine peptides by cyclization to a thiazoline for bacitracin A and by cyclization followed by dehydrogenation to thiazole rings for the others. These advances and the extent of our knowledge of the biosynthesis of the thiazolidine ring of penicillin (cf. Arnstein, 1957) have emphasized the lack of information available on the biosynthesis of the thiazole moiety (III) of thiamine, and it is with that portion of the thiamine molecule that we have concerned ourselves in particular.

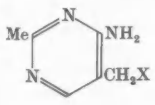
The present communication describes isotopic-competition experiments in which a range of speculative $^{32}$S-containing compounds have been studied in competition with $^{35}$S-labelled inorganic sulphate in order to determine the relative efficiencies of such compounds in making available the sulphur atom required for incorporation into the thiazole ring of thiamine during its biosynthesis in yeast. Although such experiments throw no direct light on the source of any of the carbon atoms required for the biosynthesis of thiamine, on account of the possibility of sulphur exchange at intermediate stages, they were expected to provide some guidance in the selection of compounds requiring study in the form of $^{14}$C-labelled substrates. No systematic application has hitherto been made of radioactive-tracer methods to the study of the biosynthesis of thiamine, though it has been shown that [$^{14}$C]formate is incorporated by *Bacillus subtilis* into the pyrimidine moiety, but the absence of $^{14}$C radioactivity in the thiazole moiety was taken to indicate the absence of a single-carbon-transfer system in its synthesis (Pine & Guthrie, 1959); similar observations were made with *Saccharomyces cerevisiae* (David & Estramareix, 1960). Availability of the vitamin thiazole appears to be a limiting factor in the biosynthesis of thiamine by yeast, indicating that yeast is less capable of synthesizing the thiazole moiety than it is of synthesizing the pyrimidine half of thiamine (Van Lanen, Broquist, Johnson, Baldwin & Peterson, 1942).

## MATERIALS

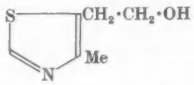
*Yeast.* Cultures were obtained from The National Collection of Yeast Cultures, The Brewing Industry Research Foundation, Nutfield, Surrey. They were maintained on agar slants at 0° and subcultured at 6-month intervals. The strain selected for use was *Saccharomyces cerevisiae* no. 1062 of the British National Collection of Yeast Cultures.




(I)




(II)




(III)

*Medium.* The basal medium contained per 100 ml. final volume (including any of the additions studied): glucose, 2·00 g.; $NH_4Cl$, 162 mg.; $KH_2PO_4$, 156 mg.; $MgCl_2,6H_2O$, 41 mg.; $CaCl_2,6H_2O$, 33 mg.; $FeCl_2,4H_2O$, 25 μg.; KI, 10 μg.; $H_3BO_3$, 10 μg.; $CuCl_2,2H_2O$, 5 μg.; $MnCl_2,4H_2O$, 4 μg.; $ZnCl_2,H_2O$, 4 μg.; $(NH_4)_6Mo_7O_{24},4H_2O$, 2 μg.; L-arginine, 53 mg.; L-leucine, 85 mg.; DL-tryptophan, 16 mg.; DL-aspartic acid, 40 mg.; calcium D-pantothenate, 500 μg.; *meso*inositol, 1 mg.; DL-biotin, 0·2 μg.

*Saline.* This was a solution of 850 mg. of NaCl in 100 ml. of water.

*Taka-diastase.* A powder preparation, stated to be 'standardized by dilution with lactose', was obtained from Parke, Davis and Co., Hounslow, Middlesex; it was used without purification as a source of phosphatase.

*Cyanogen bromide.* A concentrated solution of potassium cyanide was added slowly dropwise to saturated bromine water at 0° until decolorization was complete. The cyanogen bromide solution was used within 3 hr.

[$^{35}$S]*Sulphate.* The solution (> 100 mc/mg. of sulphur) supplied by The Radiochemical Centre, Amersham, Bucks., was used after appropriate dilution with water.

5-(2-*Hydroxyethyl*)-4-*methylthiazole* (III). Thiamine was cleaved by means of sulphite at pH 5, and the thiazole moiety was extracted with chloroform as described by Williams, Waterman, Keresztesy & Buchman (1935). The chloroform solution was then dried ($Na_2SO_4$) and fractionated, and the residue was distilled *in vacuo*.

S-*Adenosylmethionine.* Brewer's yeast (500 g.), kindly supplied by Whitbread and Co. Ltd., was enriched with DL-methionine (15 g.) and extracted according to the method of Schlenk & DePalma (1957). The final solution (140 ml.) contained 1 m-mole of *S*-adenosylmethionine; paper chromatography [Whatman no. 1 paper; ethanol–water–acetic acid (65:34:1, by vol.); ascending] gave a single ultraviolet-absorbing spot of $R_F$ 0·32. No other spot was observed under filtered ultraviolet light, or after treatment with ninhydrin or iodine vapour.

5′-*Deoxy*-5′-*methylthioadenosine.* *S*-Adenosylmethionine was hydrolysed under milder conditions than those used by Baddiley, Cantoni & Jamieson (1953). A solution (60 ml., pH 4) containing 0·5 m-mole of *S*-adenosylmethionine was heated in a steam bath for 8 hr. The mixture was then evaporated to dryness *in vacuo* and the residue was twice crystallized from water, affording 0·08 m-mole of 5′-deoxy-5′-methylthioadenosine, m.p. 204–205° (literature, m.p. 205°); paper chromatography [Whatman no. 1 paper; ethanol–water–acetic acid (65:34:1, by vol.); ascending] gave a single ultraviolet-absorbing spot of $R_F$ 0·75.

*Thiazolidine*-4-*carboxylic acid* (IV). The method of preparation from cysteine and formaldehyde described by Ratner & Clarke (1937) was followed.

DL-α-*Amino*-β-(4-*methylthiazol*-5-*yl*)*propionic acid* (V). A specimen (Harington & Moggridge, 1939) was kindly made available by Sir Charles Harington.

4-*Methylthiazole.* The method of Clarke & Gurin (1935) was followed.

*Methanethiol.* This substance was prepared by treatment of *S*-methylthiuronium sulphate with sodium hydroxide (cf. Windus & Shildneck, 1943), and ethanethiol was prepared similarly. The use of cupric chloride–charcoal traps (Hill & Wolfrom, 1947) was found expedient. The sodium derivatives were prepared by passing excess of mercaptan into an ethanolic solution of sodium ethoxide and precipitating the product with ether.

*Mercapto alcohols.* Addition of thioacetic acid to glycidol (2:3-epoxypropan-1-ol), followed by hydrolysis, gave 1-mercaptopropan-2:3-diol, b.p. 110°/7 mm., $n_D^{21}$ 1·5247, in 45% yield (Found: C, 33·2; H, 7·6. Calc. for $C_3H_8O_2S$: C, 33·0; H, 7·4%); Sjöberg (1942) gave b.p. 100–101°/1 mm., b.p. 112°/3 mm., $n_D^{20}$ 1·5268–1·5271.

Reaction between sodium thioacetate and 1-acetyl-1-chloropropyl 3-acetate and hydrolysis of the product gave 1-acetyl-1-mercaptopropan-3-ol (Matsukawa, Iwatsu & Yurugi, 1948).

*Mercaptoacetone.* Chloroacetone and NaSH afforded mercaptoacetone, m.p. 102–108°, in 75% yield under the conditions described by Jacini, Bacchetti & Rosnati (1952) and Ohta (1950); these authors gave m.p. 109° and 105–110° respectively.

4-*Amino*-2-*methyl*-5-*thioformamidomethylpyrimidine* (II; X = NH·CHS). The 5-aminomethylpyrimidine was thioformylated under the conditions described by Würsch (1958).

## METHODS

*Preparation of sulphur-deficient inoculum.* The selected strain of yeast was subcultured several times in the basal medium without any addition of sulphate or other sulphur-containing compound, and then stored, if necessary, at 0°.

*Isotopic-competition experiments.* Each experiment employed eight 1 l. flasks, each charged with a total of 100 ml. of basal medium supplemented with equal amounts (200–400 μc, based on nominal radioactivity as supplied) of [$^{35}$S]sulphate. Seven flasks received supplements of the $^{32}$S-containing compounds under test (Table 2, column 1) in known amounts between 1 and 100 μmoles (Table 2, column 3), and the eighth flask was kept as control; occasionally two controls were used. As a further control 5-(2-hydroxyethyl)-4-methyl[$^{32}$S]thiazole was tested at least once per experiment. The medium was adjusted to pH 5 if necessary, and autoclaved at 10 lb./in.$^2$ for 20 min., the glucose and [$^{35}$S]sulphate solutions being sterilized separately and then added; if the substance under test was heat-sensitive or volatile it was sterilized separately by Seitz filtration of its aqueous solution. About 5 mg. of washed sulphur-deficient yeast cells suspended in sterile saline was added to each flask, and the inoculated media (total, 100 ml. each) were then incubated at 25° with reciprocal shaking (60 excursions/min.) until little further growth occurred (about 60 hr.). The dry weight of cells (Table 2, column 4) obtained was estimated nephelometrically at 500 mμ with standard curves.

*Radioactivity of yeast cells.* Yeast suspension (0·1 ml.) was evaporated on a 1 cm.$^2$ polythene disk containing lens tissue, and the radioactivity was measured at 'infinite thinness' with an end-window Geiger counter. Under these conditions 1 μmc gave about 200 counts/min. Corrections were applied for background and dead time, and the counter was standardized with a 1 cm.$^2$ 'infinitely thick' disk containing [$^{14}$C]polymethacrylate of specific activity 1 μc/g., which gave $10^3$ counts/min. The cells were spun off and the radioactivity of 0·1 ml. of the supernatant was similarly measured. The difference between the radioactivity of the suspension and that of the corresponding supernatant gave a measure of the radioactivity incorporated into the cells (Table 2, column 5); about 90% of the available radioactivity was normally so incorporated.

*Extraction of thiamine.* The cells were washed with 50 ml. of saline and then added to a boiling mixture of 2 ml. of

0·1 N-HCl and 5 ml. of water. This ensured rapid inactivation of yeast phosphatases, as recommended by Westenbrink, van Dorp & Gruber (1941). After gentle boiling for 2 min. the suspension was cooled and centrifuged. The cell debris contained no thiamine. The supernatant was percolated down an 8 cm. × 0·5 cm. diameter column of washed Amberlite CG-50 (100–200 mesh) in the sodium form; this retained any free thiamine present at this stage, but normally there was none. The effluent and washings were pooled (approx. 10 ml.) and treated with about 0·1 ml. of M-sodium acetate buffer, pH 4·7, about 5 mg. of Takadiastase and a few drops of toluene. On incubation overnight at 40° almost quantitative hydrolysis of the cocarboxylase to thiamine took place, and the resulting solution (pH 4·7) was percolated down a 8 cm. × 0·5 cm. diameter column of washed Amberlite CG-50 (100–200 mesh) in the $H^+$ form. The effluent and washings (approx. 20 ml.) contained almost no thiamine, and were rejected; the column was then eluted with 5 ml. of 0·1 N-HCl, to give a 'standard' extract, containing over 95 % of the thiamine originally present in the yeast.

*Estimation of thiamine.* A method developed from that of Fujiwara & Matsui (1953) was used. The 'standard' yeast extract (1 ml.), containing 0·01–2 $\mu$g. of thiamine, was treated with 1 ml. of cyanogen bromide solution and then 0·5 ml. of aq. 50 % NaOH solution. *iso*Butanol (5 ml.; or 1 ml. for samples of low thiamine content) was added and the mixture was shaken vigorously for 90 sec. After centrifuging by hand the clear upper layer was pipetted into cuvettes, and fluorescence was measured with a Farrand fluorimeter and a quinine sulphate solution (434·3 $\mu$g./l. in 0·01 M-$H_2SO_4$) as standard. Drying of the *iso*butanol solution was unnecessary; the weight of thiochrome was estimated by reference to standard curves, and the total weight of thiamine extracted from the cells was calculated (Table 2, column 6). No blank reading was necessary. The thiamine content of unit weight of cells was also calculated for comparison purposes (Table 2, column 7).

*Radioactivity of thiamine.* Estimates of the radioactivity of the biosynthesized thiamine were obtained by observing the radioactivity of the derived thiochrome. *iso*Butanol solution (0·2 ml.) containing a known weight of thiochrome was transferred to a 2 cm.$^2$ polythene disk containing lens tissue, and one drop of acetic acid was added. After desiccation the radioactivity of the solute was measured as described above. The specific activity of the thiamine (Table 2, column 8) was then calculated; it is compared with that for a corresponding control (Table 2, column 9) to give the 'relative specific activity' as a percentage (Table 2, column 10).

## RESULTS

*Reliability of assay method.* The thiamine in the 'standard' extract accounted for only about 10 % of the total radioactivity of the extract, but the *iso*butanol solution of the derived thiochrome gave a single radioactive spot on paper chromatograms. It was therefore considered desirable to assess the reliability of the method by studying the radioactivity of the thiochrome obtained after the application of different methods of purification to the thiamine, and, by comparison with the results of an independent method for the assay of the specific radioactivity of the biosynthesized thiamine, to permit the selection of conditions suitable for routine use.

Yeast was grown under the standard conditions described above in the presence of (i) [$^{35}$S]sulphate, and (ii) [$^{35}$S]sulphate plus 5-(2-hydroxyethyl)-4-methyl[$^{32}$S]thiazole (1 $\mu$mole/100 ml. of medium); the inoculum in both cases consisted of about 4 mg. of washed yeast cells, and the dry weight of cells at the end of 5 days was 29 mg. in both cases. 'Standard' extracts (each 5 ml.) were prepared from the washed cells and processed side by side in the following ways: (*a*) 1 ml. portions of the 'standard' extracts were treated with cyanogen bromide, and the specific activities of the thiamine estimated as thiochrome. The remaining 4 ml. portions of the 'standard' extract were treated with 50 $\mu$g. of [$^{32}$S]thiamine and freeze-dried. The residues were taken up in 80 % ethanol and each

Table 1. *Specific activities of biosynthesized* [$^{35}$S]*thiamine after various treatments of the 'standard' extract of yeast grown in the presence of* (i) [$^{35}$S]*sulphate and* (ii) [$^{35}$S]*sulphate plus* 5-[2-*hydroxyethyl*]-4-*methyl*[$^{32}$S]*thiazole*

Paper chromatography: Whatman no. 1; ethanol–water–acetic acid (65:34:1, by vol.), ascending flow. Paper electrophoresis: Whatman no. 54; 10 % formic acid under $CCl_4$; 27–33 v/cm.; 45 min.

| Treatment of 'standard' extract | Form assayed | Specific activity of biosynthesized thiamine (counts/min./$\mu\mu$mole) (i) | (ii) |
|---|---|---|---|
| (*a*) None; thiamine oxidized directly | Thiochrome | 37·3 | 8·7 |
| (*b*) Dilution with thiamine; sulphite cleavage of thiamine; 5-[2-hydroxyethyl]-4-methylthiazole picrate crystallized to constant radioactivity | 5-[2-Hydroxyethyl]-4-methylthiazole picrate | 37·3 | 7·4 |
| (*c*) Dilution with thiamine; freeze-dried; thiamine oxidized directly | Thiochrome | 37·3 | 10·9 |
| (*d*) Dilution with thiamine; paper chromatography as thiamine | Thiochrome | 37·3 | 3·4 |
| (*e*) Dilution with thiamine; paper electrophoresis as thiamine | Thiochrome | 37·6 | 4·0 |
| (*f*) Thiochrome from (*b*) chromatographed and recovered | Thiochrome | 32·0 | 7·4 |
| (*g*) Thiochrome from (*f*) subjected to electrophoresis and recovered | Thiochrome | 31·6 | 9·7 |

solution was divided into four portions, which were treated in pairs in the following ways: (*b*) dilution with 50 mg. of [$^{32}$S]thiamine, cleavage with sulphite and assay as 5-(2-hydroxyethyl)-4-methylthiazole picrate, recrystallized to constant radioactivity; (*c*) direct conversion into thiochrome and assay as before; (*d*) paper chromatography, automatic scanning of chromatogram for radioactivity, elution, conversion into thiochrome, and assay; (*e*) paper electrophoresis, automatic scanning for radioactivity, elution, conversion into thiochrome, and assay. In addition, the thiochrome obtained in (*c*) was (*f*) chromatographed on paper, eluted and again assayed, and the recovered thiochrome was then (*g*) subjected to electrophoresis on paper, eluted and again assayed. The results are recorded in Table 1.

The excellent agreement between the values for the specific activities of biosynthesized thiamine given in (i) (*a*)–(*e*) showed that direct oxidation of thiamine in the 'standard' extract and assay as thiochrome was adequate for routine use. The fluctuations of specific activity observed in experiment (ii) are attributable to the lower counts obtained in that experiment. The lower specific activities observed in (i) (*f*) and (i) (*g*) may have been due to some further purification of the thiochrome, or, more probably, to some instrumental error in view of the good agreement shown between (i) (*a*) and (i) (*b*)–(*e*).

*Isotopic competition experiments*

Yeast uses [$^{35}$S]sulphate efficiently as a source of sulphur for the biosynthesis of thiamine, as shown by Table 1, Expt. (i), and if the medium also contains the [$^{32}$S]thiazole moiety of thiamine the specific activity of the biosynthesized thiamine is substantially decreased, as shown by Table 1, Expt. (ii). The presence of a non-radioactive sulphur source in the medium will result in a lower specific activity of biosynthesized thiamine only if: (*a*) the non-radioactive sulphur compound is absorbed by the yeast and transported, either as such or in a modified form, to the appropriate site(s) inside the cell where required; (*b*) the sulphur atom, with or without attached carbon atoms, is then available for incorporation into the thiamine molecule. Although the method can only be expected to detect substances capable of providing the sulphur atom of thiamine, if a significantly low specific activity is observed the compound under test must satisfy conditions (*a*) and (*b*) and may in addition be a possible precursor of the thiazole moiety of thiamine; in this way the selection of possible $^{14}$C-labelled compounds suitable for further studies could be facilitated.

For convenience we define the relative specific activity due to a compound as the ratio (expressed as a percentage) of the specific activity of thiamine from yeast grown in a medium containing [$^{35}$S]-sulphate and a given concentration of the particular $^{32}$S-labelled compound to that of the thiamine from yeast grown in an identical 'control' medium containing no non-radioactive sulphur competitor; the figures so obtained give a measure of the efficiency with which conditions (*a*) and (*b*) are met. Table 2 gives the results of experiments designed to determine the relative specific activities due to various non-radioactive sulphur-containing compounds at different concentrations. In three cases (Expts. 1–3) where controls were carried out in duplicate, the reproducibility of the experiment was satisfactory, variations of 6–8% being observed.

*Sulphate and 5-(2-hydroxyethyl)-4-methylthiazole.* When $Na_2{}^{32}SO_4$ is used as non-radioactive competitor the effect is to lower the specific activity of the [$^{35}$S]sulphate in the medium, and conditions (*a*) and (*b*) must both be fulfilled equally well by both species. Non-radioactive sulphate at 1 $\mu$mole/100 ml. resulted in a relative specific activity of 62%, and 10 $\mu$moles/100 ml. in a relative specific activity of 7%. These figures correspond to a control medium containing about 1 $\mu$mole of total available sulphur/100 ml.; this would include sulphur transferred to the medium with the inoculum (which would depend on the history of the inoculum) and impurities in the medium, as well as the radioactive sulphur (less than 0·2 $\mu$mole/100 ml.) added. The amount of [$^{35}$S]sulphate varied from experiment to experiment between 200 and 400 $\mu$c, but was constant throughout any one experiment, and each experiment included a control. The concentration in the medium of available sulphur from all sources was not accurately known, but the concentration of adventitious sulphur-containing material was kept as low as possible and as constant as practicable; nevertheless the specific activity of the thiamine isolated from the controls varied over a considerable range (34–106 counts/min./$\mu\mu$mole), reflecting changes in the available sulphur content of the media, and, in particular, in the ratio of $^{35}$S to $^{32}$S. These fluctuations had, however, no effect on the relative specific activities as defined above; thus the relative specific activities of thiamine obtained from cultures containing 1 $\mu$mole of the non-radioactive thiazole/100 ml. of medium were relatively constant at 20–29%, confirming that the relative specific activity values obtained for the same substance in the same concentration but in different experiments are comparable. By inference, therefore, the relative specific activities of thiamine biosynthesized in the presence of different compounds in different experiments may be compared in assessing the relative efficiencies of various com-

pounds as sources of the sulphur atom for the biosynthesis of thiamine.

If the effects of 1 $\mu$mole of the thiazole and of [$^{32}$S]sulphate/100 ml. are compared, it is apparent that the thiazole is about twice as efficient as sulphate in the provision of the sulphur atom for thiamine biosynthesis. At concentrations of 10 $\mu$moles/100 ml., however, the relative specific activity due to the thiazole does not change, whereas that due to [$^{32}$S]sulphate falls below the expected value of 14%, based on the value of the relative specific activity observed for 1 $\mu$mole/100 ml. Whereas the sulphate must continue to fulfil conditions (*a*) and (*b*), the additional thiazole evidently cannot meet condition (*a*), since if all the thiazole were available for thiamine biosynthesis a fall in relative specific activity to about 3% would result. Biosynthesis directly from sulphate of about one-quarter of the total thiamine therefore occurs when the medium contains 500 or even 5000 times the amount of the thiazole required for full incorporation. On the other hand, if only 0·05 $\mu$mole of the thiazole/100 ml. (equivalent to 25 times the weight of thiamine biosynthesized) was provided, the thiamine obtained had a relative specific activity of 40%, implying a greater efficiency of incorporation at higher dilution. These results emphasize the importance of condition (*a*) and the inefficiency (relative to sulphate) of transport of the thiazole to the thiamine-producing sites in the cell; they also confirm that the thiazole is incorporated by the yeast into the thiamine molecule.

Although it is generally believed that sulphate must first be reduced before the sulphur can become available for biosynthetic processes in yeast, sodium sulphide appeared to be less effective than sodium sulphate at equivalent concentrations.

*Methionine, homocysteine, homocystine, cysteine.* It is evident from the values for relative specific activity recorded in Table 2 that methionine is more efficient than cysteine, homocysteine or homocystine in providing the sulphur atom of thiamine. In spite of the known metabolic interrelationships, the differences in comparable relative specific activities are significant (DL-methionine: 14, 13; DL-homocysteine: 50; DL-homocystine: 97%. L-Methionine: 15, 5, 10; L-cysteine, 78, 83, 85, 69%). In comparing the values for relative specific activity due to DL- and to L-methionine, it seems probable that the D-enantiomorph has little effect. These and other comparisons suggest that the sulphur atom is incorporated into thiamine most directly from L-methionine. Homocysteine is less efficient as a source of sulphur, and methylation to methionine may be an obligatory stage. The inefficiency of homocystine may be due to inefficient transport, since, once inside the cell, rapid reduction to homocysteine would be expected. It is well known that disulphides are reduced to thiols by metabolizing yeast (Neuberg & Schwenk, 1915), and it has been reported by Sandegren, Ekström & Nielsen (1950) that in the yeast *Rhodotorula gracilis* growing on a limited sulphur supply all the polarographically active sulphur present is in the thiol form. These results run roughly parallel with the observations on protein synthesis in yeast (*Torulopsis utilis*) by Roberts, Cowie, Abelson, Bolton & Britten (1955), who found cysteine to be a more effective competitor than cystine and also noted the relative inefficiency of homocysteine and the greater inefficiency of homocystine, which they attributed to inability of the yeast cell to reduce the disulphide forms.

Cysteine is also much less efficient than homocysteine or methionine, suggesting that it can provide sulphur for incorporation into thiamine only via the cystathionine–homocysteine–methionine pathway. Djenkolic acid (relative specific activity, 108%) was without effect, and the relative specific activity due to ethionine could not be determined, since the presence of this amino acid in the medium inhibited yeast growth.

*Glutathione.* Comparison of the relative specific activity values due to sulphate, glutathione and cysteine at 1 $\mu$mole/100 ml. (62, 57 and 85% respectively) or at 10 $\mu$moles/100 ml. (7, 5 and 69–85% respectively) shows that the two former compounds can supply the sulphur atom required for thiamine biosynthesis much more efficiently than can cysteine, and the provision of glutamic acid and glycine as well as cysteine in the medium did not affect the relative specific activity appreciably. Since glutathione is biosynthesized from L-cysteine the marked difference in their competitive powers is surprising.

*S-Adenosylmethionine and 5′-deoxy-5′-methylthioadenosine.* The success with which methionine competed with sulphate as the source of the sulphur for incorporation into the thiamine molecule prompted the examination of *S*-adenosylmethionine and of 5′-deoxy-5′-methylthioadenosine; both entered into competition with sulphate, the former slightly more efficiently and the latter less efficiently than methionine.

*Thiazolidine-4-carboxylic acid* (IV). Neither cysteine nor 4-methylthiazole gave relative specific activities for biosynthesized thiamine, suggesting that they were of significance in yeast, but the values for relative specific activity obtained with thiazolidine-4-carboxylic acid showed that it acted as a sulphur donor as efficiently as methionine; by comparison with the inefficiency of cysteine this result was unexpected. The more direct observations of Korte, Weitkamp & Vogel (1959), using

Table 2. *Relative specific activities of thiamine biosynthesized by yeast in isotopic-competition experiments between 200–400 μc of [$^{35}$S]sulphate/100 ml. and various compounds containing non-radioactive sulphur*

| Non-radioactive-sulphur-containing competitor (1) | Expt. no. (2) | Concn. of competitor in medium (μmoles/100 ml.) (3) | Dry wt. of harvested cells (mg./100 ml.) (4) | Total radioactivity of cells (mega-counts/min.) (5) | Wt. of thiamine isolated* (μm-moles) (6) | Thiamine content of cells* (μg./g.) (7) | Sp. activity of thiamine* (counts/min./μμmole) (8) | Sp. activity of corresponding control (counts/min./μμmole) (9) | Relative sp. activity of biosynthesized thiamine (%) (10) |
|---|---|---|---|---|---|---|---|---|---|
| None† | 1 | 0 | 41 | 55·1 | 1·37‡ | — | 43·3 } 44·6 | — | 97 |
| | 1 | 0 | 41 | 62·0 | 2·17 | 18 | 45·9 } (av.) | — | 103 |
| | 2 | 0 | 37 | 54·0 | 1·97 | 18 | 45·9 } 44·3 | — | 104 |
| | 2 | 0 | 37 | 59·6 | 1·78 | 17 | 42·7 } (av.) | — | 96 |
| | 3 | 0 | 20 | 52·1 | 1·10 | 19 | 106·1 } 103·0 | — | 103 |
| | 3 | 0 | 22 | 51·7 | 1·10 | 17 | 99·8 } (av.) | — | 97 |
| | 4 | 0 | 30 | 68·4 | 1·81 | 21 | 51·8 | — | — |
| | 5 | 0 | 29 | 56·9 | 2·02 | 24 | 48·8 | — | — |
| | 6 | 0 | 24 | 40·4 | 1·04 | 15 | 89·2 | — | — |
| | 7 | 0 | 32 | 41·7 | 1·31 | 14 | 57·0 | — | — |
| | 8 | 0 | 24 | 40·9 | 1·14 | 16 | 60·1 | — | — |
| | 9 | 0 | 32 | 58·8 | 2·30 | 25 | 38·0 | — | — |
| | 10 | 0 | 22 | 44·3 | 1·53 | 24 | 45·8 | — | — |
| | 11 | 0 | 15 | 27·4 | 0·95 | 20 | 51·5 | — | — |
| | 12 | 0 | 12 | 28·0 | 0·87 | 20 | 55·7 | — | — |
| | 13 | 0 | 22 | 17·2 | 1·39 | 22 | 34·6 | — | — |
| Sodium sulphate | 8 | 1 | 40 | 41·8 | 2·15 | 19 | 37·4 | 60·1 | 62 |
| | 3 | 10 | 122 | 71·5 | 13·15 | 37 | 7·3 | 103·0 | 7 |
| 5-(2-Hydroxyethyl)-4-methylthiazole | 1 | 0·05 | 41 | 57·4 | 2·37 | 20 | 17·8 | 44·6 | 40 |
| | 1 | 1 | 41 | 59·9 | 2·37 | 20 | 8·9 | 44·6 | 20 |
| | 3 | 1 | 22 | 56·6 | 1·10 | 17 | 29·7 | 103·0 | 29 |
| | 3 | 1 | 22 | 57·9 | 1·18 | 16 | 24·5 | 103·0 | 24 |
| | 6 | 1 | 22 | 39·9 | 1·04 | 18 | 22·2 | 89·2 | 25 |
| | 7 | 1 | 30 | 39·3 | 1·58 | 24 | 13·2 | 57·0 | 23 |
| | 9 | 1 | 33 | 56·9 | 2·31 | 19 | 8·8 | 38·0 | 23 |
| | 10 | 1 | 21 | 45·0 | 1·39 | 22 | 11·1 | 45·8 | 24 |
| | 11 | 1 | 14 | 27·1 | 0·78 | 19 | 12·1 | 51·5 | 24 |
| | 12 | 1 | 12 | 29·0 | 0·88 | 19 | 11·8 | 55·7 | 21 |
| | 2 | 10 | 41 | 58·2 | 2·11 | 18 | 10·4 | 44·3 | 23 |
| | 8 | 10 | 26 | 40·7 | 1·14 | 15 | 15·1 | 60·1 | 25 |
| | 13 | 10 | 26 | 17·7 | 1·44 | 19 | 7·3 | 34·6 | 21 |
| DL-Methionine | 1 | 2 | 53 | 60·5 | 3·51 | 23 | 27·2 | 44·6 | 61 |
| | 9 | 2 | 51 | 61·5 | 3·57 | 24 | 25·4 | 38·0 | 67 |
| | 9 | 20 | 115 | 70·0 | 10·55 | 32 | 5·4 | 38·0 | 14 |
| | 10 | 20 | 89 | 40·1 | 9·40 | 36 | 5·8 | 45·8 | 13 |
| L-Methionine | 5 | 1 | 43 | 57·5 | 3·15 | 25 | 34·7 | 48·8 | 71 |
| | 2 | 10 | 106 | 72·4 | 6·85 | 22 | 6·8 | 44·3 | 15 |
| | 7 | 10 | 85 | 40·4 | 4·94 | 20 | 2·8 | 57·0 | 5 |
| | 8 | 10 | 144 | 47·9 | 11·27 | 27 | 6·3 | 60·1 | 10 |
| DL-Homocysteine | 13 | 20 | 39 | 19·9 | 2·60 | 23 | 17·3 | 34·6 | 50 |
| | 11 | 100 | 106 | 32·4 | 8·83 | 29 | 4·2 | 51·5 | 8 |
| DL-Homocystine | 1 | 2 | 41 | 58·1 | 2·56 | 22 | 41·9 | 44·6 | 94 |
| | 13 | 10 | 25 | 17·9 | 1·39 | 19 | 32·9 | 34·6 | 95 |
| | 5 | 20 | 36 | 54·2 | 2·37 | 23 | 47·3 | 48·8 | 97 |
| | 8 | 100 | 47 | 41·9 | 3·01 | 22 | 31·1 | 60·1 | 52 |
| L-Cysteine | 1 | 1 | 41 | 60·2 | 2·67 | 23 | 37·8 | 44·6 | 85 |
| | 2 | 10 | 37 | 57·4 | 2·07 | 19 | 34·7 | 44·3 | 78 |
| | 8 | 10 | 29 | 39·8 | 1·37 | 16 | 50·0 | 60·1 | 83 |
| | 11 | 10 | 18 | 25·0 | 1·16 | 20 | 43·6 | 51·5 | 85 |
| | 13 | 10 | 41 | 19·4 | 2·49 | 21 | 23·9 | 34·6 | 69 |
| | 12 | 100 | 40 | 23·7 | 2·86 | 25 | 19·4 | 55·7 | 35 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 11 | 10 | 41 | 19·4 | 2·49 | 21 | 23·9 | 34·6 | 69 | |
| | 13 | 10 | 41 | 19·4 | 2·49 | 25 | 19·4 | 55·7 | 35 | |
| | 12 | 100 | 40 | 23·7 | 2·86 | | | | | |

Table 2 (*cont.*)

| Non-radioactive-sulphur-containing competitor (1) | Expt. no. (2) | Concn. of competitor in medium ($\mu$moles/100 ml.) (3) | Dry wt. of harvested cells (mg./100 ml.) (4) | Total radioactivity of cells (mega-counts/min.) (5) | Wt. of thiamine isolated* ($\mu$m-moles) (6) | Thiamine content of cells* ($\mu$g./g.) (7) | Sp. activity of thiamine* (counts/min./$\mu\mu$mole) (8) | Sp. activity of corresponding control (counts/min./$\mu\mu$mole) (9) | Relative sp. activity of bio-synthesized thiamine (%) (10) |
|---|---|---|---|---|---|---|---|---|---|
| Djenkolic acid | 4 | 10 | 30 | 64·7 | 2·07 | 24 | 56·0 | 51·8 | 108 |
| Glutathione | 1 | 1 | 49 | 62·1 | 3·11 | 22 | 25·5 | 44·6 | 57 |
| | 3 | 10 | 117 | 71·2 | 12·57 | 37 | 5·4 | 103·0 | 5 |
| | 10 | 10 | 73 | 49·4 | 8·10 | 38 | 3·9 | 45·8 | 8 |
| Glutamic acid+cysteine+glycine | 11 | 10 each | 17 | 26·7 | 1·07 | 19 | 45·3 | 51·5 | 88 |
| *S*-Adenosylmethionine | 9 | 2 | 65 | 60·6 | 5·26 | 28 | 15·1 | 38·0 | 40 |
| | 9 | 20 | 147 | 67·9 | 14·31 | 34 | 3·0 | 38·0 | 8 |
| 5′-Deoxy-5′-methylthioadenosine | 9 | 2 | 47 | 64·0 | 3·34 | 25 | 30·5 | 38·0 | 80 |
| | 9 | 20 | 104 | 64·3 | 8·67 | 29 | 11·5 | 38·0 | 30 |
| Thiazolidine-4-carboxylic acid | 13 | 1 | 28 | 19·1 | 1·88 | 23 | 22·8 | 34·6 | 66 |
| | 13 | 10 | 58 | 17·7 | 6·13 | 37 | 3·8 | 34·6 | 11 |
| | 12 | 100 | 128 | 29·3 | 10·7 | 29 | 0·6 | 55·7 | 1 |
| 4-Methylthiazole | 12 | 100 | 17 | 29·4 | 1·53 | 26 | 37·3 | 55·7 | 67 |
| | 13 | 10 | 34 | 18·8 | 2·14 | 22 | 22·2 | 34·6 | 63 |
| DL-α-Amino-β-(4-methylthiazol-5-yl)propionic acid | 6 | 2 | 22 | 43·9 | 0·88 | 14 | 68·5 | 89·2 | 77 |
| | 6 | 20 | 24 | 35·9 | 1·06 | 15 | 36·9 | 89·2 | 41 |
| | 7 | 20 | 33 | 41·2 | 1·14 | 12 | 20·5 | 57·0 | 36 |
| (4-Methylthiazol-5-yl)acetic acid | 1 | 1 | 41 | 60·7 | 2·31 | 19 | 43·4 | 44·6 | 97 |
| Benzylpenicillin | 10 | 100 | 25 | 45·1 | 1·47 | 20 | 43·5 | 45·8 | 95 |
| DL-Biotin | 8 | 20 | 26 | 40·7 | 1·23 | 16 | 59·8 | 60·1 | 100 |
| Methanethiol | 5 | 1 | 43 | 64·3 | 3·24 | 26 | 38·5 | 48·8 | 79 |
| | 5 | 10 | 51 | 54·2 | 3·76 | 26 | 33·0 | 48·8 | 68 |
| | 2 | 100§ | 106 | 58·2 | 14·3 | 47 | 1·4 | 44·3 | 3 |
| Sodium methyl sulphide | 6 | 100§ | 68 | 44·1 | 6·33 | 32 | 23·5 | 89·2 | 26 |
| | 7 | 100§ | 79 | 41·1 | 4·16 | 18 | 15·7 | 57·0 | 28 |
| Ethanethiol | 4 | 10 | 36 | 69·6 | 4·42 | 42 | 18·6 | 51·8 | 36 |
| Sodium ethyl sulphide | 6 | 100 | 78 | 45·5 | 4·86 | 22 | 14·0 | 89·2 | 16 |
| *S*-Methylthiuronium chloride | 4 | 10 | 30 | 65·0 | 2·04 | 24 | 54·6 | 51·8 | 105 |
| Thiourea | 1 | 1 | 39 | 57·4 | 2·07 | 18 | 46·0 | 44·6 | 103 |
| Thioacetic acid | 4 | 10 | 11·5 | 44·9 | 1·39 | 42 | 17·0 | 51·8 | 33 |
| | 10 | 10 | 37 | 35·9 | 3·70 | 34 | 14·7 | 45·8 | 32 |
| Thioglycollic acid | 4 | 10 | 30 | 66·0 | 2·60 | 30 | 46·5 | 51·8 | 90 |
| Taurine | 2 | 1 | 41 | 64·4 | 2·01 | 17 | 40·7 | 44·3 | 92 |
| 1:2-Dimercaptopropan-3-ol (BAL) | 5 | 10 | 31 | 49·8 | 2·25 | 25 | 40·2 | 48·8 | 82 |
| 1-Mercaptopropan-2:3-diol | 7 | 100 | 27 | 37·3 | 1·70 | 22 | 49·5 | 57·0 | 87 |
| | 8 | 100 | 28 | 38·6 | 1·23 | 15 | 38·4 | 60·1 | 64 |
| 1-Acetyl-1-mercaptopropan-3-ol | 6 | 100 | 22 | 46·1 | 1·42 | 22 | 45·2 | 89·2 | 51 |
| 4-Amino-2-methyl-5-thioformamidomethyl-pyrimidine | 11 | 10 | 25 | 27·5 | 2·31 | 34 | 33·6 | 51·5 | 65 |
| | 12 | 100§ | 50 | 33·5 | 6·70 | 45 | 13·8 | 55·7 | 25 |
| Sodium sulphide | 4 | 10 | 53 | 66·0 | 5·36 | 35 | 21·6 | 51·8 | 42 |
| Thiamine | 2 | 0·24 | 46 | 59·6 | 64·2 | 483 | 0·3 | 44·3 | 1 |
| | 4 | 0·003 | 22 | 57·8 | 1·92 | 30 | 38·0 | 51·8 | 73 |

* Estimated as thiochrome. † Controls for numbered experiments. ‡ Some yeast accidentally lost. § Concentration uncertain owing to volatility and/or insolubility.

4-methyl[2-$^{14}$C]thiazole, showed that that substance remained unchanged in cultures of a range of micro-organisms capable of effecting partial synthesis of thiamine.

DL-α-*Amino*-β-(4-*methylthiazol*-5-*yl*)*propionic acid* (V). The observed relative specific activity values due to the DL-amino acid confirm utilization by yeast to some extent for thiamine biosynthesis, but, when compared with the corresponding values due to 5-(2-hydroxyethyl)-4-methylthiazole itself, suggest that the acid does not lie on the normal route, since 1 μmole of the DL-amino acid/100 ml. (relative specific activity of biosynthesized thiamine, 77 %) is much less efficient than an equivalent concentration of 5-(2-hydroxyethyl)-4-methylthiazole (relative specific activity of biosynthesized thiamine, 25 %).

4-Methylthiazol-5-ylacetic acid had no effect on thiamine biosynthesis.

*Benzylpenicillin*. The relative specific activity of thiamine (97 %) biosynthesized in the presence of 100 μmoles of benzylpenicillin/100 ml. of medium shows that it is ineffective as a source for the supply of the sulphur atom of thiamine.

*Biotin*. The standard medium always contained about 1 μm-mole of DL-biotin/100 ml., which was essential for growth. When it was supplemented with 20 μmoles there was little change in the relative specific activity of the thiamine (91 %), showing that the sulphur of biotin is not available as a source of sulphur for incorporation into thiamine.

*Alkanethiols*. The thiols were either added to the medium as such or as their solid sodium derivatives; in either case some thiol was lost. Tight cotton-wool plugs and cupric chloride-impregnated charcoal traps were used, and any thiol in the gas phase was continuously shaken up with the yeast suspension. The concentration of thiol available in the medium was therefore uncertain, and the figures given in Table 2 relate to the quantities of thiol initially added. Nevertheless, it is apparent from the relative specific activity values that both thiols compete with sulphate for the provision of the sulphur required for thiamine biosynthesis. Neither *S*-methylthiuronium chloride nor thiourea had any significant effect.

*Miscellaneous compounds*. At a concentration of 10 μmoles/100 ml. thioacetic acid ($CH_3 \cdot CO \cdot SH$) gave a relative specific activity of 32–33 % for biosynthesized thiamine showing some degree of incorporation of its sulphur atom, possibly by way of a thioacetylation mechanism; thioglycollic acid ($HS \cdot CH_2 \cdot CO_2H$) at the same concentration was almost ineffective (relative specific activity of biosynthesized thiamine, 89 %), as also was taurine.

Other compounds tested were 1:2-dimercaptopropan-3-ol (dimercaprol, BAL), 1-mercaptopropan-2:3-diol and 1-acetyl-1-mercaptopropan-3-ol, which might have been capable of conversion into the thiazole moiety of thiamine. Although effects were observed in the presence of large amounts, in no case did the relative specific activity of biosynthesized thiamine fall to a level indicating noteworthy incorporation of sulphur from these sources. The presence of mercaptoacetone in the medium inhibited yeast growth, and no reliable relative specific activity values could be obtained.

The thioformyl derivative of the pyrimidine moiety of thiamine [4-amino-2-methyl-5-thioformamidomethylpyrimidine (II; X = NH·CHS)] gave values of relative specific activity for biosynthesized thiamine indicating utilization of the sulphur to some extent; this might have been due to partial decomposition during incubation and subsequent utilization of the fragments, as is also observed in pea roots (Bonner & Buchman, 1938).

## DISCUSSION

As the strain of yeast selected for use was non-exacting (B. L. Brady, personal communication, 1957) it was expected to be capable of synthesizing all its sulphur compounds from inorganic sulphate, and when given [$^{35}$S]sulphate it was found to synthesize thiamine of high specific activity, the only dilution suffered by the carrier-free radioisotope coming from the limiting amount of endogenous sulphur present in the inoculum and in the medium, not all of which would be expected to be available for thiamine biosynthesis. The specific activity of the biosynthesized thiamine was in fact of the order of 1 mc/mg., compared with about 120 μc/mg. for thiamine synthesized from [$^{35}$S]-thiourea (Würsch, 1958), so that biosynthesis affords a convenient method for the preparation of small amounts of [$^{35}$S]thiamine of high specific activity. The amount of thiamine synthesized by yeast, however, is extremely small and most of the sulphur is used for other purposes; on the other hand [$^{35}$S]sulphate is relatively cheap. When grown in the synthetic medium described, the yeast normally contained about 25 μg. of thiamine (as cocarboxylase) per gram (dry weight) of cells (Table 2, column 7); this figure is within the range quoted for non-enriched yeasts (Van Lanen & Tanner, 1948). The thiamine content of the yeast could be increased only by providing thiamine or both its moieties in the medium; in these circumstances less thiamine was biosynthesized, and it incorporated less label, though it was present in larger quantities. This was demonstrated by the use of thiamine itself as non-radioactive competitor for the [$^{35}$S]sulphate, when both the specific and total activities of the extracted thiamine were lower than those of controls. Moreover, not all the

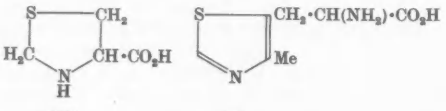
added vitamin could be accounted for, suggesting that some of it had been metabolized (cf. Bonner & Buchman, 1939; Tatum, 1944). No advantage would therefore be gained in the routine use of thiamine-enriched yeast.

In a typical experiment, 250 $\mu$c of [$^{35}$S]sulphate was added to 100 ml. of the basal medium; the 25 mg. of yeast obtained had incorporated 220 $\mu$c, of which about 25 $\mu$c was extracted by the boiling acid. A radioactivity of 5 $\mu$c was retained by the first chromatographic treatment and 15 $\mu$c was not retained by the second. The 5 ml. of 'standard' extract contained 5 $\mu$c of which [$^{35}$S]thiamine accounted for about 0·5 $\mu$c. Since 25 mg. of yeast cells contained only about 0·5 $\mu$g. of thiamine, its specific activity had to be high to enable its total activity to be measured. The specific activity of the [$^{35}$S]sulphate in the medium had therefore to be high; a basal medium was therefore chosen which contained no sulphur other than added [$^{35}$S]sulphate and sulphur intrinsically present in the inoculum. The use of [$^{35}$S]sulphate of high activity (> 100 mc/mg. of sulphur), and of small volumes of inoculum consisting of washed yeast cells, which were themselves sulphur-starved, minimized introduction of extraneous sulphur, and yielded thiamine whose specific activity was conveniently measurable. Preliminary experiments confirmed the observations of Kleinzeller, Kotyk & Kováč (1959) that both glucose and ammonium salts must be present in the medium for maximum uptake of sulphate by non-proliferating yeast. Under these conditions [$^{35}$S]thiamine is found in non-proliferating cells kept in the presence of [$^{35}$S]sulphate, demonstrating thiamine turnover, but the label is necessarily diluted by the non-radioactive sulphur already present in the yeast.

Cowie, Bolton & Sands (1951) have demonstrated that the presence of non-radioactive sulphur compounds, in a medium containing [$^{35}$S]sulphate and enough total sulphur for optimum growth, may eliminate or suppress uptake of the label by *Escherichia coli* cells. The presence of a sulphur-containing compound in a medium containing little sulphur as sulphate (about 1 $\mu$mole/100 ml.) in which sulphur-starved yeast is growing may well have other effects than on incorporation of the extra sulphur into the thiamine of the yeast; indeed the general sulphur metabolism in such yeast may be abnormal. Table 2, column 5, gives the total activity of cells grown in such a medium and shows that the effect observed with *E. coli* is not obvious with yeast. Variations in the specific activity of biosynthesized thiamine are therefore not due to selective uptake of sulphur compounds by the yeast cell. It is likely that all the non-radioactive compounds tested are also absorbed by the cell and are there available to compete more or less efficiently with [$^{35}$S]sulphate, but it is not known to what extent selective transport occurs within the cell, and it is in this respect that the competition may not be a fair one. The major proportion of a competitor might remain near the cell wall while the [$^{35}$S]sulphate reached thiamine-producing sites relatively unhindered. In this connexion the localization of *S*-adenosylmethionine in enriched *Torulopsis utilis* cells is of interest; this yeast stores large quantities of 'active methionine' in the vacuole (Svihla & Schlenk, 1959; 1960). The addition of some $^{32}$S-containing compounds caused greatly enhanced yeast growth (Table 2, column 4), thus confirming their utilization by the yeast, but hindering comparisons. The thiamine content of unit weight of yeast, however, did not vary widely (Table 2, column 7), confirming the inability of the yeast to synthesize and store extra thiamine unless provided with the vitamin itself or its moieties. It may be that the extra vitamin (much of which is stored as free thiamine) is stored without turnover and is not in equilibrium with thiamine biosynthesized *de novo*. One & Ichikawa (1954) have suggested that exogenous thiamine is not absorbed through the cell wall, and electron microscopy indicates that it is located on the cell wall (Ichikawa, 1956).

The isotopic-competition methods described here do not allow rigorous quantitative treatment of the results, and even qualitative interpretation may be misleading. Nevertheless, in the group of sulphur-containing amino acids, L-methionine appeared to be about twice as effective as the DL-form, which, in turn, was much more effective than DL-homocysteine in supplying the sulphur atom for incorporation into the thiazole moiety of thiamine. Fukui (1950) reported that *Mucor racemosus* can form thiamine from 4-amino-5-aminomethyl-2-methylpyrimidine (II, X = $NH_2$) and methionine in the Reader medium, whereas *Aspergillus oryzae* and *Saccharomyces saké* can also do so if the medium is supplemented with peptone, so that methionine appears to be a significant precursor. The fact that efficiencies as sulphur donors for thiamine biosynthesis appeared to follow the order homocysteine < methionine < *S*-adenosylmethionine > 5′-deoxy-5′-methylthioadenosine seems to indicate that *S*-adenosylmethionine is more directly concerned with the biosynthesis than are the other members of the series. Nakayama (1956) has reported that mutant strains of *Escherichia coli* and *Neurospora crassa*, which both require 5-(2-hydroxyethyl)-4-methylthiazole (III) for growth, responded moderately to 4-methylthiazole; cysteine and thiazolidine-4-carboxylic acid (IV) maintained the growth of both strains, but no nutritive activity was observed in homocysteine, methionine or methionol. As thiamine and 5-(2-hydroxyethyl)-4-methyl-

thiazole were detected in *Neurospora* cultures that had been incubated with cystine or thiazolidine-4-carboxylic acid, it was assumed (Nakayama, 1956) that biosynthesis of thiamine took place from cystine via thiazolidine-4-carboxylic acid and 4-methylthiazole. In yeast, however, only thiazolidine-4-carboxylic acid appeared to be of significance as a sulphur donor, and it was comparable with methionine in efficiency. The apparent inefficiency of cysteine and the effectiveness of glutathione are at first sight difficult to reconcile with each other, but it may be a reflexion of a wider range of uses by yeast for cysteine, and the same weighting factor no doubt applies with other substrates to unknown and varying extents.



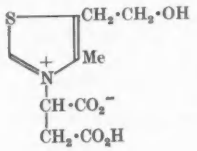
(IV) (V)

α-Amino-β-(4-methylthiazol-5-yl)propionic acid (V) has been suggested as a precursor of 5-β-hydroxyethyl-4-methylthiazole (Harington & Moggridge, 1939; Buchman & Richardson, 1939) and it could be derived from methionine, acetaldehyde and ammonia (Harington & Moggridge, 1939). Homocysteine has been postulated as a precursor of the thiazole moiety of thiamine in a *Neurospora* mutant, possibly by conversion into *S*-formylhomocysteine, which could condense with a –C.C.N– fragment to give the same amino acid (V) (Doudney & Wagner, 1953). It has been shown that the L-form of this amino acid is converted into 5-(2-hydroxyethyl)-4-methylthiazole (III) by brewer's yeast, leaving the D(−)-form unchanged (Harington & Moggridge, 1940); conversion into 5-(2-hydroxyethyl)-4-methylthiazole also occurs in pea roots (Bonner & Buchman, 1938) but not in *Phycomyces blakesleeanus* (Bonner, quoted by Buchman & Richardson, 1939), in *Staphylococcus aureus* (Knight, quoted by Harington & Moggridge, 1939), or in *Neurospora crassa* (Tatum & Bell, 1946), as the amino acid cannot replace 5-(2-hydroxyethyl)-4-methylthiazole in these systems. In yeast it would not appear that the amino acid (V) lies on the direct route of thiamine biosynthesis, although it is utilized to some extent. Bonner & Buchman (1938) also found that pea roots could apparently synthesize thiamine when given its pyrimidine moiety and (i) 5-(2-hydroxyethyl)-4-methylthiazole, or (ii) thioformamide and 1-acetyl-1-chloropropan-3-ol, or (iii) thioformamide and 1-acetylpropan-3-ol. Tatum & Bell (1946), however, showed that such mixtures [(ii) and (iii)] could not replace 5-(2-hydroxyethyl)-4-methylthiazole in an exacting mutant strain of *Neurospora*, and Louis (1951) found isolated pea roots capable of synthesizing thiamine from the intact pyrimidine and thiazole moieties but not from the pyrimidine, thioformamide and 1-acetyl-1-chloropropan-3-ol. *Mucor racemosus*, *Saccharomyces saké* and *Serratia marcescens* are said to synthesize thiamine from 4-amino-5-formamidomethyl-2-methylpyrimidine (II; X = NH·CHO) and 1-acetyl-1-mercaptopropan-3-ol, and *M. racemosus*, *S. saké* and *Bacillus vulgatus* are said to produce thiamine from the formamidomethylpyrimidine and 1-acetyl-1-chloropropan-3-ol (Fukui & Kishibe, 1951). It has been pointed out, however, that, some of these mixtures may give thiamine *in vitro* under mild conditions without enzymic intervention (Matsukawa & Yurugi, 1951; Bonner & Buchman, 1938), and the components appear unlikely ones in natural biosynthesis. Neither 4-amino-2-methyl-5-thioformamidopyrimidine nor 1-acetyl-1-mercaptopropan-3-ol appeared to be capable of contributing sulphur effectively for thiamine biosynthesis in yeast, as shown by the high concentrations necessary to affect appreciably the relative specific activities of biosynthesized thiamine in the present work.

A number of cases have been described in which a substance other than the vitamin thiazole (III) has appeared to be capable of replacing the latter in growth tests on what have been regarded as exacting organisms, but it is difficult to assess the significance of such growth tests. For example, penicillin in $10^5$-fold concentration is capable of replacing 5-(2-hydroxyethyl)-4-methylthiazole as a growth factor for *Phycomyces blakesleeanus* (Shulman, Dick & Farrer, 1957), and a similar effect has been described for bacitracin, particularly for specimens liable to contain bacitracin F (Ebringer, 1960). There can be no question of conversion of these substances into the vitamin thiazole, and benzylpenicillin is certainly incapable of supplying the sulphur atom required for thiamine biosynthesis.

The fairly inefficient use of methane- and ethanethiol as sources of the sulphur atom in thiamine is of interest. It has been reported that methane- and ethane-thiol are incorporated into *S*-adenosylmethionine and *S*-adenosylethionine by yeast (*Torulopsis utilis*) (Schlenk, 1957; Schlenk & Tillotson, 1954); on the other hand, it has also been reported that methane[$^{35}$S]thiol is converted by yeast into *S*-methyl[$^{35}$S]cysteine and that the major known sulphur compounds, such as cysteine, glutathione and methionine, do not become labelled (Black, Downey & Wolff, 1957). The comparable behaviour of methane- and ethane-thiol in the present experiments would seem to indicate that a process of sulphur exchange operates, and the relative efficiencies as competitors of the various

compounds examined may be a measure of the relative abilities of the yeast to metabolize these substances to give a common, and at present unidentified, sulphur-containing intermediate in the biosynthesis of thiamine. Apart from the vitamin thiazole itself, 5-(2-hydroxyethyl)-4-methylthiazole, none of the compounds examined can be recognized as a major precursor of the thiazolium ring of thiamine. The mechanism by which these compounds that can compete with sulphate make sulphur available for biosynthesis of thiamine is by no means clear; whether carbon is simultaneously incorporated from any of these compounds can only be determined by experiments with $^{14}C$-labelled materials.




(VI)

In conclusion, the prosthetic group of crystalline rabbit-muscle α-glycerophosphate dehydrogenase has properties which suggest that it has the quaternary thiazole structure (VI); it can replace the thiazole moiety of thiamine in growth tests on *Leuconostoc mesenteroides*, and thiamine deficiency in rats results in lowering of soluble glycerophosphate dehydrogenase (van Eys, 1960). What may be the relationship of this substance, which has been named thiamic acid, to the thiazole moiety of thiamine remains to be seen.

## SUMMARY

1. Yeast grown on a defined medium containing [$^{35}S$]sulphate produces [$^{35}S$]thiamine of high specific activity in small amounts.

2. Yeast has been grown in a defined medium containing [$^{35}S$]sulphate and a non-radioactive sulphur-containing substance as sole sulphur sources. These two substances may, or may not, compete in contributing the sulphur required for incorporation into the thiazole ring of thiamine; the specific activity of the biosynthesized thiamine compared with controls gives a measure of the efficiency of the non-radioactive sulphur-containing compound under test as a donor of sulphur for the biosynthesis of thiamine.

3. The following compounds are utilized by the yeast, in the presence of sulphate, as sources of sulphur for the biosynthesis of thiamine: very efficiently: 5-(2-hydroxyethyl)-4-methylthiazole; efficiently: *S*-adenosylmethionine, sodium sulphate, glutathione, methionine, thiazolidine-4-carboxylic acid, 5′-deoxy-5′-methylthioadenosine; fairly efficiently: α-amino-β-(4-methylthiazol-5-yl)propionic acid, sodium sulphide, thioacetic acid; inefficiently: cysteine, homocysteine, 4-amino-2-methyl-5-thioformamidomethylpyrimidine, methanethiol and ethanethiol.

4. Other compounds tested which do not appear to compete significantly with sulphate are: homocystine, thiourea, biotin, thioglycollic acid, djenkolic acid, *S*-methylthiuronium chloride, 4-methylthiazole, (4-methylthiazol-5-yl)acetic acid, benzylpenicillin, taurine, 1:2-dimercaptopropan-3-ol (BAL), 1-mercaptopropan-2:3-diol, 1-acetyl-1-mercaptopropan-3-ol.

5. Of the compounds examined, none—apart from 5-(2-hydroxyethyl)-4-methylthiazole—could be recognized as a major precursor of the thiazolium ring in thiamine.

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# Metabolism of Collagen

## APPEARANCE AND DISAPPEARANCE OF [$^{14}$C]HYDROXYLYSINE IN RAT CONNECTIVE TISSUE

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The technique of labelling collagen with the radioisotope of a distinctive amino acid, hydroxylysine, simplifies the study of collagen metabolism. In collagen from adult animals this amino acid is derived from the hydroxylation of lysine after the 'parent' amino acid has been incorporated into a polypeptide (Sinex & Van Slyke, 1955, 1957; Sinex, Van Slyke & Christmas, 1959).

The metabolism of collagen has previously been studied by isotopically labelling a constituent amino acid. [$^{14}$C]Glycine was used in investigations on the rabbit corium (Harkness, Marko, Muir & Neuberger, 1954) and by Jackson (1957) on the water- and salt-soluble and -insoluble fractions of the carageenin granuloma. However, glycine, though present in high concentrations in collagen,

does not specifically identify this fibrous protein. Green & Lowther (1959) studied collagen synthesis by following the conversion of [$^{14}$C]proline into [$^{14}$C]hydroxyproline in slices of carageenin granuloma. Problems germane to the isolation and purity of collagen are eliminated by the use of either the proline–hydroxyproline or the lysine–hydroxylysine system.

This paper presents the results of an investigation into the metabolism of [$^{14}$C]hydroxylysine-labelled collagen in the developing tissue *in vivo* of adult rats of both sexes.

## METHODS

Connective tissue was obtained from adult male and female Sprague–Dawley rats (6–18 months of age) by the sponge-implant technique (Boucek & Noble, 1955). Three sponges were implanted aseptically along the dorsum in the loose subcutaneous fascia. All animals were maintained on Purina Laboratory Chow given *ad lib.*

The amount of [$^{14}$C]hydroxylysine in the sponge implants of 8, 12, and 20 days was determined. Seventy animals of each sex were treated, and the sponge tissues were removed at specified intervals (0·5, 1·0, 2·0, 3·0, 4·5, 6·0, 8·0, 9·5, 12·0, 14·5 and 16·0 hr.) after a single intraperitoneal injection of DL-[2-$^{14}$C]lysine monohydrochloride (Tracerlab Inc., Waltham, Mass., U.S.A.; 12·5 $\mu$c/100 g. body wt.; $2{\cdot}8 \times 10^6$ counts/min. in 21 $\mu$moles dissolved in 0·5 ml. of 0·15 M-NaCl, pH 7·4).

Immediately after removal, the implant was freed of surrounding fascia and weighed. The tissue was then homogenized (VirTis Macrohomogenizer, The VirTis Co. Inc., Yonkers, New York) in 7 ml. of cold 0·45 M-NaCl (pH 7·4) for 0·5 min. at minimum and maximum speeds, transferred to a centrifuge tube and made to a volume of 25 ml. Centrifuging at low speed separated the fibres, and deoxyribonucleic acid (DNA) was determined on 0·5 ml. of the supernatant solution (Ceriotti, 1952). The mixture from the centrifuge tube was then placed in Lusteroid tubes and shaken for 24 hr. After centrifuging for 1 hr. at 55 000*g* (Spinco L preparative ultracentrifuge), the salt extract was removed. Three additional 24 hr. extractions with twice the tissue volume of 0·45 M-NaCl (pH 7·4) were made (Gross, 1958). The combined salt extracts and the residue were dialysed against redistilled water until free of salt. All extraction and dialysis procedures were performed at 4° and in the presence of octan-2-ol. The two tissue fractions were dried, and collagen was extracted with hot (90°) trichloroacetic acid (Fitch, Harkness & Harkness, 1955).

Tissues from 25 animals of each sex were not subjected to salt extraction but were minced and extracted directly with trichloroacetic acid.

The trichloroacetic acid extracts were dried and subsequently hydrolysed for 16 hr. in 6 N-HCl at 110° in sealed tubes. A portion of the hydrolysed sample was used for hydroxyproline determination (Neuman & Logan, 1950) and column chromatography after it had been dried three times to remove HCl and neutralized to pH 7·0.

Separation of the basic amino acids was effected by elution from a Dowex 50 X 8 (200–400 mesh) column by the procedure of Moore & Stein (1951). The neutral and acidic acids were eluted with citrate buffer (pH 5·0) and the three basic amino acids, histidine, hydroxylysine and lysine, with phosphate buffer (pH 6·8). The phosphate buffer was collected in fractions (2 ml.) at the rate of 6 ml./hr. A portion (1 ml.) of each eluate fraction was dried on an aluminium planchet for measurement of radioactivity, and the amino acid concentration of the remaining 1 ml. was determined by the ninhydrin technique (Moore & Stein, 1954). The planchets were counted in a gas-flow counter with a thin-window Geiger–Müller tube. Corrections were made for background radioactivity and for the counting efficiency of the instrument. The thickness of the sample was such that the count obtained did not require a correction for self-absorption. The standard error calculated for the number of counts accumulated was 3·2–7·1 % (Boucek, Noble & Woessner, 1959*a*).

The collagen content of 7-, 8-, 12- and 20-day implants from 139 animals was calculated from the amount of hydroxyproline in the tissue, with the conventional conversion factor of 7·46 (Neuman & Logan, 1950).

Blood samples were taken by cardiac puncture at intervals starting at 15 min. after the intraperitoneal injection. The radioactivity of the deproteinized serum (counts/min./ml.) was converted into lysine on the basis of the determined specific activity of the injected [$^{14}$C]lysine.

*Calculations.* Radioactivity of hydroxylysine was expressed in terms of either its specific activity (counts/min./mg.), its relation to cells (counts/min./mg. of DNA) or its content (counts/min./g. of sponge). The amounts of hydroxyproline (collagen) and DNA in the tissue were calculated in relation to the dry weight of the implanted sponge. The effect of variations in water content or non-collagenous substances of the tissue upon the data was thus eliminated.

DNA content (mg./g. of sponge) of 6–20-day tissues from 46 female and 32 male rats was plotted against tissue age, and the 'best-fit' lines were determined by the method of least squares. The value for any tissue age was obtained from the derived line.

## RESULTS AND DISCUSSION

After the implantation of the sponge into the loose subcutaneous tissue, the interior becomes filled with blood elements, including leucocytes. It has been observed in histological sections that the leucocytes found during the first 7 days are ultimately replaced by fibroblasts (Boucek, 1959). The initial amount of DNA in the implant decreases until the sixth day (Boucek, Noble & Woessner, 1959*b*). This finding agrees with the histological observations of a decrease in leucocytes during the early period. From the sixth to the twentieth day the content of DNA increased linearly (Fig. 1), the amount in the female being greater than in the male. The cellular population during this period has been shown histologically to be made up principally of invading fibroblasts.

At present, the factors which stimulate the pre-existing fibrocytes to proliferate at the site of an injury are not fully understood. It has been observed, however, that even during the first

3 days after implantation, the concentration of DNA was greater in the female. This initial nucleic acid concentration was due to leucocytes and, accepting the premise that the products of leucocytes stimulate the production of fibroblasts (Carrel, 1922), the sex difference in the concentration of DNA in the 6–20-day tissue may be explained in part by the initial leucocytic response.

*Appearance of* [$^{14}$C]*lysine in the serum and of* [$^{14}$C]*hydroxylysine in the tissue.* The maximum observed activity of the serum occurred 15 min. after the injection and the value was unaltered 15 min.

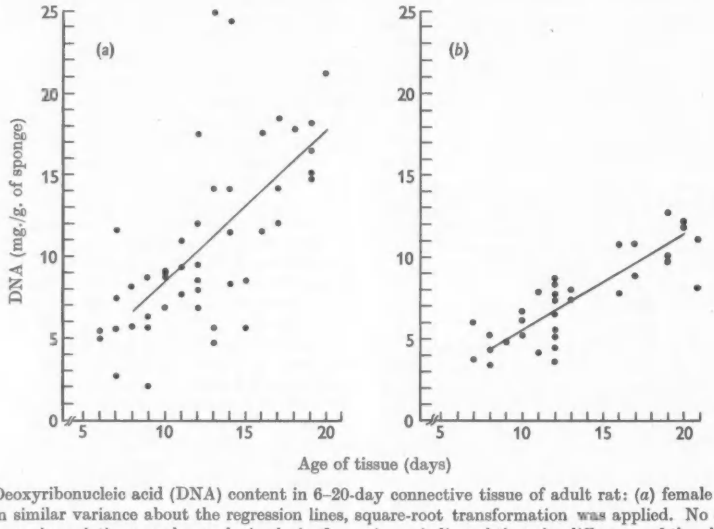


Fig. 1. Deoxyribonucleic acid (DNA) content in 6–20-day connective tissue of adult rat: (*a*) female; (*b*) male. To obtain similar variance about the regression lines, square-root transformation was applied. No significant sex difference in variation was observed. Analysis of covariance indicated that the difference of the elevation of the lines, best estimated as 3·8 mg. of DNA/g. of sponge, was significant ($P < 0.001$).

Table 1. *Appearance of* [$^{14}$C]*lysine in serum and of* [$^{14}$C]*hydroxylysine in tissue*

Numbers of animals are shown in parentheses in the second column. Means and standard deviations of the serum radioactivities are given.

| Time after injection (min.) | Serum (counts/min./ml.) | Tissue fraction | |
|---|---|---|---|
| | | Salt-soluble (counts/min./mg. of hydroxylysine) | Salt-insoluble |
| 15 | 50 398±2 864 (4) | — | — |
| 30 | 48 278±12 102 (11) | 4 854 | 121 |
| | | 5 403 | 57 |
| | | 5 657 | 55 |
| | | 5 632 | — |
| 60 | 25 260±12 430 (9) | 9 323 | 135 |
| | | 6 494 | 143 |
| | | 7 093 | 155 |
| | | 9 593 | 77 |
| | | | 143 |
| 90 | — | 2 621 | 280 |
| | | 11 834 | 375 |
| | | 6 088 | 198 |
| | | | 171 |
| 180 | 20 861 | 10 843 | 749 |
| | 11 315 | 6 534 | 678 |
| | 9 555 | 3 240 | 267 |
| | 7 038 | — | 770 |
| | | | 772 |

later (Table 1). The activity then decreased rapidly to a level which remained constant from the third to the eighth hour. Sex did not influence this response.

The appearance of [$^{14}$C]hydroxylysine in tissue from the 12-day implants of both sexes was measured during the 16 hr. after the intraperitoneal injection. Specific-activity data for the two sexes were combined because the amounts of collagen and labelled hydroxylysine were generally similar at this tissue age. Labelled hydroxylysine in a non-dialysable form appeared in the salt-soluble fraction within 30 min.; the specific activity of the hydroxylysine in this fraction continued to increase for a further 30 min. (a time when the specific activity of the serum was already declining), after which there was considerable variability (Table 1). A decline of the specific activity in the salt-soluble fraction occurred after the third hour, undoubtedly reflecting the diminished concentration of the serum [$^{14}$C]lysine.

As early as 30 min. after injection, a small amount of labelled hydroxylysine appeared in the salt-insoluble fraction. However, 60 min. after injection, a rapid accumulation of the labelled hydroxyamino acid began, at a rate of 258 counts/min./mg./hr. After the third hour, the rate slowed, due in part to the decline in the activity of the salt-soluble fraction.

*Sex difference in the appearance of* [$^{14}$C]*hydroxylysine.* The 1–3 hr. rate of synthesis of [$^{14}$C]-hydroxylysine collagen expressed on a cellular basis (counts/min./mg. of DNA/hr.) was usually greater in the male (Fig. 2). Similar rates occurred only in the 12-day tissue. These results indicated that the cells from the male generally synthesize collagen more rapidly.

That such a sex difference has validity is established by the following facts: (1) The serum supply of free labelled lysine during the 1–3 hr. period was the same in both sexes, approx. 5·4 mg./100 ml. at the peak concentration (obtained by the procedure described in the Methods section). This concentration alone is more than twice the reported value for free serum lysine of the rat (Wu, 1954). (2) The vascular supply to the tissues appears to be similar on histological examination. An indirect measurement of vascularity is provided by haemoglobin determinations on tissue homogenates by Woessner in our laboratories (Flink & Watson, 1942). The haemoglobin value increased with tissue age, but was similar in both sexes. (3) The concentration of hydroxylysine in collagen from the sponge tissue of the two sexes was similar. Mature collagen is characterized by an hydroxylysine concentration of 1·3 g./100 g. (Bowes & Kenten, 1948), although younger forms of collagen, as reported by Van Slyke & Sinex (1958), have a lower concentration (0·7 g./100 g.). In the 8-day sponge tissue of both sexes, a concentration of 0·65 g./100 g. was found in the trichloroacetic acid extracts of the insoluble fraction. At the twelfth and twentieth days the hydroxylysine of the collagen in this fraction increased to 0·8 g./100 g. (4) The ratio, hydroxylysine:hydroxyproline, in the salt-soluble and -insoluble collagen was not different for the two sexes.

The sex difference in the rates of synthesis was also evident in the 12 hr. kinetic study of the appearance of labelled hydroxylysine in young connective tissue. With this technique of intraperitoneal administration of [$^{14}$C]lysine, the labelled 8- and 12-day collagen of both sexes was found to reach maximum value between the eighth and the twelfth hours. A maximum value did not occur until 16 hr. in the 20-day tissues (Table 2). However, it was not possible to derive a meaningful kinetic expression for the appearance of [$^{14}$C]-hydroxylysine beyond the third hour. Factors such as the decline in [$^{14}$C]lysine activity of the serum, the decrease in the concentration of salt-soluble [$^{14}$C]hydroxylysine and the process of collagen degradation contribute to the departure of the data from the linear expression of the 1–3 hr. period.

It was apparent from the peak concentration of the isotope that the 8-day tissue from the male had an extraordinary capacity for retention of a large amount of [$^{14}$C]hydroxylysine (Table 2). The 8-day tissue from the female contained fibroblasts with a lower rate of synthesis but the cells were more numerous. The [$^{14}$C]hydroxylysine of the 8–12 hr. peak therefore should have been greater than or equal to that of the male. However, the value from the male tissue appeared to be greater ($P < 0·05$), although the variability was great at this period. Obviously, some of the factors mentioned above, probably the process of collagen degradation, are

Table 2. [$^{14}$C]*Hydroxylysine in young connective tissue*

Numbers of animals are shown in parentheses. Means and standard deviations of the tissue radioactivities are given.

| | | Adult rat (counts/min./g. of sponge) | |
|---|---|---|---|
| Time after injection (hr.) ... | | 8–12 | 16 |
| | Age of tissue (days) | | |
| Male | 8 | 2432±813 (5) | 1564±682 (6) |
| | 12 | 2008±600 (8) | 1665±754 (12) |
| | 20 | 1043±195 (3) | 1726±426 (5) |
| Female | 8 | 1352±529 (5) | 1151±306 (6) |
| | 12 | 2641±253 (6) | 1103±202 (10) |
| | 20 | 904 (2) | 1003±199 (5) |

more active in the tissue from the female at this age. The resultant of collagen synthesis minus degradation is the net gain, and at 8 days a sex difference occurred. The collagen concentration (hydroxyproline conversion) was $113 \pm 70$ mg./g. of sponge in eight tissues from the male whereas ten tissues from the female had $57 \pm 33$ mg./g. of sponge ($P < 0{\cdot}05$).

The peak quantity of [$^{14}$C]hydroxylysine of the 12-day tissue was greater in the female ($P < 0{\cdot}05$, Table 2). Since the rates of synthesis were similar until this age, this larger quantity is undoubtedly attributable to the more numerous cells in the female.

*Disappearance of* [$^{14}$C]*hydroxylysine.* Only a part of the synthesized [$^{14}$C]hydroxylysine remained in the 8- and 12-day tissues. That this new collagen had a high rate of turnover was clearly indicated by the rapid disappearance of labelled hydroxylysine between the peak value and the sixteenth hour (Table 2). By the twentieth day this highly labile collagen was not detected.

Whereas collagen synthesis could be related to cellular activity, its disappearance could not. For example, no relationship was apparent between the 16 hr. value of [$^{14}$C]hydroxylysine collagen and the concentration of DNA. At the three tissue ages of the developing connective tissue of each sex, the 16 hr. concentrations of labelled collagen were similar in spite of an increasing concentration of DNA.

This similarity of the 16 hr. values strongly suggests the formation of constant amounts of collagen resistant to degradation and presumably in a crystalline state. This form of collagen was stable, remaining in the tissues for weeks.

The production of large amounts of collagen in the early stages of fibrosis probably lends stability to the initial gel formation of wound healing. In addition, this young collagen probably exists in a less well-organized state than does the highly crystalline mature collagen. The more random state of young collagen would undoubtedly enhance its susceptibility to degradation.

The finding of collagens with dissimilar metabolic properties was not surprising, for Harkness & Moralee (1956) have found uterine collagen to be rapidly metabolized. Jackson (1957) has reported on the extremely active synthesis and degradation of collagen in the carageenin granuloma.

The process of collagen degradation is not well understood. In the post-parturient uterus of the rat, collagen rapidly decreases within 2–3 days. Woessner (1959) reports the breakdown of collagen to the amino acid level in the post-parturient uterus, indicating the presence of active proteolytic enzymes. He has also noted the presence of large concentrations of proteases and peptidases in the connective tissue of the implant (Woessner & Boucek, 1959). Older collagen, such as that of the corium, 'turns over' quite slowly, having almost an infinite half-life (Neuberger & Slack, 1953). However, the complete mechanism of collagen breakdown remains to be determined.

*Sex differences in the disapperance of* [$^{14}$C]*-hydroxylysine.* In the 8- and 12-day tissues of both sexes, when large amounts of collagen were being synthesized, the disappearance of [$^{14}$C]hydroxylysine was rapid. The gradual accumulation of labelled collagen for 16 hr. in the 20-day tissue to a value approximating that remaining in the young tissue after active degradation indicates that the older tissue synthesized a collagen which probably polymerized directly into a crystalline form.

One consistent sex difference occurred in the 16 hr. values (counts/min./g. of sponge). The mean for 23 male tissues 16 hr. after the injection was 1600 counts/min./g. of sponge whereas the mean value for 21 female tissues was 1100 ($P < 0{\cdot}001$). Conditions apparently exist in the male tissue which favour the formation of crystalline, more resistant collagen.

Factors which influence the crystalline state of collagen are unknown. Banfield (1952, 1956) reported a quantitative decrease in acid-soluble collagen and a decrease in the swelling of human Achilles tendon, and Kohn & Rollerson (1958) noted a decrease in acid-solubility of diaphragm-tendon collagen as a function of age. In 120-day human sponge-biopsy connective tissue from young, middle-aged and old donors, a difference in swelling with acetic acid occurred (Elden, Noble & Boucek, 1959). A host effect on this process was suggested, and these results were interpreted to indicate an increase in the degree of cross-linking in collagen with advancing age. The nature of these added cross-links is unknown, but their additions produce a greater crystallinity, which would resist swelling, acid-solubilization and digestion with collagenase (Keech, 1954). Presumably, it is the state of crystallinity in collagen which affects the tissue degradation of [$^{14}$C]hydroxylysine and the amount of resistant collagen (16 hr. value).

*Collagen accumulation.* After the insertion of the implant, collagen accumulates at a decreasing rate in the newly formed connective tissue of the rat. Previously reported data indicated that collagen accumulation in the female reached a maximum value of 246 mg./g. of sponge by the fortieth day. In the male, collagen accumulation continued beyond 100 days to a value of 344 mg./g. of sponge (Kao, Boucek & Noble, 1957; Noble, Woessner, Kao & Boucek, 1958; Boucek *et al.* 1959*a*, *b*).

Seven days after implantation, the concentration of collagen varied considerably: $18 \pm 13$ mg./g. of sponge (mean value for tissues from 10 female

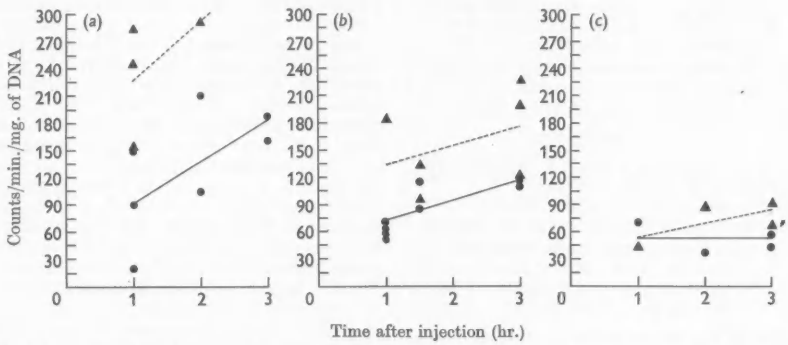


Fig. 2. Collagen synthesis in connective tissue of the rat. At each tissue age, rates were calculated from at least four points during the 1–3-hr. period after injection of [$^{14}$C]lysine: (*a*) 8-day tissue; (*b*) 12-day tissue; (*c*) 20-day tissue; ● (—), female; ▲ (- - -), male.

and 14 male rats). The kinetic studies of [$^{14}$C]-hydroxylysine–collagen synthesis indicated a rapid rate at 8 days in both sexes. However, the net gain of collagen by the eighth day appeared to be less in the tissue from the female. This suggests a greater lability of collagen in the female and the formation of a larger proportion of the stable form by the male. A rapid gain in collagen occurred in the female by the twelfth day. This may be correlated with the enhanced rate of synthesis by the 12-day cells in the females (Fig. 2) and with a less rapid breakdown than in the 8-day implants. The mean concentration after 12 days was $133 \pm 28$ mg./g. of sponge in 33 female and $149 \pm 34$ in 34 male rats. By 20 days, the mean concentration in 13 tissues from the female was $164 \pm 27$, and in 17 tissues from the male the concentration was $195 \pm 64$.

## SUMMARY

1. Collagen metabolism in developing connective tissue of the adult rat was studied by the techniques of sponge-implantation and the specific labelling of collagen with [$^{14}$C]hydroxylysine. This labelling was introduced by intraperitoneal administration of [$^{14}$C]lysine, which reached its maximum observed serum concentration within 15 min. The labelled lysine was hydroxylated in the newly synthesized peptide destined for collagen.

2. Young connective tissue was characterized by the synthesis of a large amount of labile collagen. Stable collagen was formed either as a part of the labile-collagen synthesis or independently. This stable form was constant in amount at 8, 12 and 20 days' tissue age and remained in the tissue for weeks. The extremely labile form of collagen was synthesized only during the early period of connective-tissue formation. At 20 days, synthesis of this form of collagen was not detected.

3. The metabolism of collagen was influenced by the sex of the host. Isotopic studies indicated a more active synthesis of collagen and a formation of larger amounts of stable collagen by the cells of the male. These characteristics should effect a greater accumulation of collagen in the tissue from the male.

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# The Hydrolysis of Phenyl Phosphate by Mouse-Liver Acid Phosphatase

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To determine acid-phosphatase activities of liver homogenates, with phenyl phosphate as substrate, previous workers have usually adopted methods similar to the Robinson, Gutman & Gutman (1939) adaptation of the King & Armstrong (1934) method for serum phosphatase (see, for example, Greenstein, 1942; Kochakian & Fox, 1944; Abul-Fadl & King, 1949; Goodlad & Mills, 1957). However, results obtained by Tsuboi (1952), in studies of the hydrolysis of phenyl phosphate by mouse-liver acid phosphatase, have indicated that the assay conditions of the Robinson *et al.* (1939) method, and of similar methods, allow only partial expression of the capacity of liver homogenate to hydrolyse phenyl phosphate in acid media.

In the present work, a re-examination has been made of the hydrolysis of phenyl phosphate by mouse-liver acid phosphatase and, although the results have differed from those of Tsuboi (1952), it has been confirmed that the Robinson *et al.* (1939) procedure is unsuitable for quantitative measurement of liver acid-phosphatase activity. From the results of these studies a simple and rapid method for quantitative measurement of mouse-liver acid-phosphatase activity with phenyl phosphate has been developed.

## MATERIALS AND METHODS

Experimental animals were normal, well-fed, adult male mice of mixed laboratory strains. Chemicals were of A.R. grade whenever possible. Spectrophotometric measurements were made on a Unicam spectrophotometer (SP. 500) and pH measurements on a model C pH electrometer (N. L. Jones, Melbourne). Enzyme assays and homogenate incubation were made at 37° with shaking racks fitted to a Braun model V rotary Warburg bath.

*Phenol solution.* A stock phenol soln. (1 g./l.), prepared by dissolving distilled phenol in N-HCl, was standardized by iodometric titration (Cameron & White, 1940). $E_{290} - E_{320}$ and $E_{295} - E_{320}$ values, for mM-phenol in N-NaOH, were 2·46 and 1·89 respectively. Corresponding values of 2·45 and 1·92 were reported by Brawerman & Chargaff (1954).

*Phenyl phosphate.* Commercial disodium phenyl phosphate was purified by the method of Tsuboi (1952). Total phosphorus of product, 14·3%; theoretical for $Na_2C_6H_5PO_4$, 14·2%.

*Ethylenediaminetetra-acetate.* The commercial disodium salt of ethylenediaminetetra-acetic acid (EDTA) was purified by the method of Blaedel & Knight (1954).

*Sulphydryl compounds.* *p*-Chloromercuribenzoic acid was prepared according to Whitmore & Woodward (1941). Phenyl mercuric acetate was obtained from British Drug Houses Ltd., and *o*-iodosobenzoic acid from L. Light and Co. Ltd. Aqueous solutions of these compounds were prepared with minimal NaOH.

Fresh solutions of L-cysteine hydrochloride (British Drug Houses Ltd.) were adjusted to pH 5·9 before use.

*Preparation of liver homogenates.* Preliminary studies of the intracellular distribution of mouse-liver acid phosphatase, with phenyl phosphate as substrate, indicated that mitochondria contained unreactive enzyme (equivalent to approx. 40% of the total enzyme activity) and that suspension of the mitochondria in water led to activation of this bound enzyme. These results were in general agreement with those obtained by Berthet & de Duve (1951) in studies of the hydrolysis of $\beta$-glycerophosphate by rat-liver acid

phosphatase. Water homogenates were therefore used to measure total liver acid-phosphatase activity.

Freshly excised liver, freed from gall-bladder, was homogenized in previously boiled and cooled glass-distilled water in a Potter & Elvehjem (1936) homogenizer. The homogenate (usually 0·8%, w/v) was stood in an ice bath for 15–30 min. and samples of the supernatant were used for enzyme determinations.

Total nitrogen was measured by the method of Francis, Mulligan & Wormall (1954).

*Measurement of enzyme activities.* The substrate phenyl phosphate ($pK_2$, 5·72; King & Delory, 1939) was also used as buffer (pH 5·9) except as indicated in the text. Thus freshly prepared solutions of disodium phenyl phosphate were adjusted to pH 5·9 with HCl.

To tubes containing 5·0 ml. of buffered substrate solution (at 37°), samples of homogenate (0·5 ml.) were added and incubation was continued, with shaking, at 37°. Enzyme action was arrested by addition of 1 ml. of N-NaOH. In control tubes, homogenate was added after NaOH. All determinations were run in duplicate. Phenol values were determined from measurements of $E$ at 320, 295 and 290 mμ (Brawerman & Chargaff, 1954). This method of phenol measurement offered advantages over the colorimetric method of King & Armstrong (1934) in being simpler and in being free from interference by cysteine and by EDTA.

Measurements were also made of the inorganic orthophosphate released by phosphatase action on phenyl phosphate. After spectrophotometric measurement of phenol, 2·5 ml. of 30% trichloroacetic acid was added to each tube, the tube contents were centrifuged and inorganic phosphate in the supernatant was determined by the method of King (1932). In all experiments (except in studies of the effects of inorganic phosphate and pyrophosphate on acid-phosphatase activity) phosphate values, corrected for inorganic phosphate in controls, agreed closely with values expected from phenol determinations. However, phosphate values were used only to confirm phenol values and are not reported in this paper.

Measurement of the acid-phosphatase activity of mouse blood indicated that, under the conditions used, blood in the preparations made negligible contributions to hydrolysis of phenyl phosphate by homogenates of mouse liver.

## RESULTS

### *Optimum* pH

The effect of pH on enzyme activity was determined in experiments in which liberated phenol was measured by reaction with Folin & Ciocalteu (1927) reagent (Cameron & White, 1940). A single well-defined peak of enzyme activity was obtained (Fig. 1). The optimum pH range was subsequently confirmed in experiments in which phenol was measured by ultraviolet spectrophotometry (Brawerman & Chargaff, 1954) and in which inorganic phosphate was determined by the method of King (1932).

Since the hydrolysis of phenyl phosphate tends to increase the pH of the solution (Schønheyder, 1952), substrate solutions were subsequently adjusted to pH 5·9.

### *Effect of substrate concentration*

With homogenate samples containing 1–20 mg. (wet wt.) of liver, maximum rates of hydrolysis were obtained at pH 5·9 with phenyl phosphate concentrations of approximately 0·1 M. Increasing the substrate concentration to 0·25 M did not cause enzyme inhibition.

With 0·15 M-phenyl phosphate (pH 5·9) and incubation for 5 min. periods, a linear relationship between rate of hydrolysis and quantity of liver (1–20 mg. wet wt.) was obtained.

### *Instability of liver acid phosphatase in dilute homogenates*

*In the absence of substrate.* In preliminary studies of the relationships between liver acid-phosphatase activity and incubation period it was observed that short incubation periods (5–10 min.) generally resulted in significantly higher activity values than were obtained with longer incubation periods. Since it appeared that these differences were due to enzyme inactivation during incubation at 37°, the stability of liver acid phosphatase, in both the

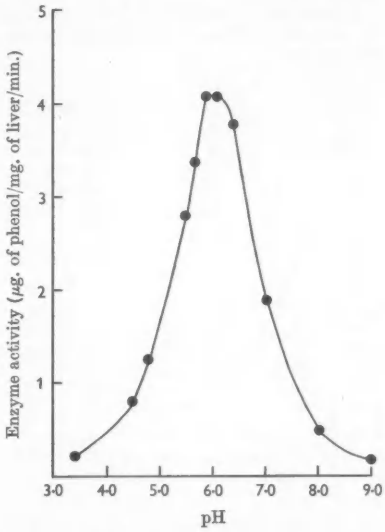


Fig. 1. pH–Activity curve of phenyl phosphate hydrolysis by mouse-liver acid phosphatase. Reaction mixtures (5·5 ml.) contained 3·5 mg. of liver and 0·14 M-sodium phenyl phosphate and were incubated for 5 min. at 37°. Buffers used: pH 3·4–4·5, 0·10 M-sodium acetate–HCl; pH 4·8–6·4, 0·14 M-disodium phenyl phosphate–HCl; pH 7·0–9·0, 0·10 M-sodium veronal–HCl. Enzyme activity was arrested with 2·5 ml. of diluted (1:3) Folin & Ciocalteu (1927) reagent and phenol measured colorimetrically (Cameron & White, 1940).

absence and the presence of substrate, was examined. Dilute, untreated liver homogenate (0·6%, w/v; pH 6·8) showed slow loss of enzyme activity when stored in an ice bath, and preincubation of the same homogenate at 37°, with shaking, resulted in increased enzyme inactivation. Adjustment of the homogenate to pH 5·9, the pH of enzyme assay, caused a further increase in the rate of inactivation at 37° (Fig. 2).

When adjustment of the pH of preincubated liver homogenate was made with sodium malonate–HCl buffer, pH 5·9, the rate of enzyme inactivation varied with malonate concentration. As shown in Fig. 3, increasing the concentration of malonate in preincubated homogenate (1 hr. at 37°) led to increased protection of enzyme until, at 0·15 M-malonate, enzyme inactivation was completely prevented.

This effect was not due simply to increase in ion content since added NaCl (0·1 M) failed to influence enzyme inactivation in the presence of 0·01 M-sodium malonate–HCl buffer and marked variations in enzyme inactivation occurred in the presence of other buffer systems. Comparisons of the effects produced by sodium malonate–HCl, sodium citrate–HCl, sodium succinate–HCl, disodium EDTA–NaOH ($pK_3$ of EDTA, 6·16; Martell & Calvin, 1952) and sodium acetate–HCl buffer systems (all 0·1 M, pH 5·9) showed that citrate was nearly as efficient as malonate in protecting acid phosphatase, that EDTA and succinate were noticeably less efficient than malonate and that acetate apparently had no protective action (Table 1). In assessing the results of Table 1, allowance was made for direct inhibition of enzyme activity by the tested buffers. With the fresh, buffered homogenates malonate was non-inhibitory whereas succinate caused 20%, citrate 9%, acetate 8% and EDTA 6% inhibition.

Possible participation of $O_2$, in the process of enzyme inactivation, was indicated by the finding that enzyme inactivation was slightly reduced when $N_2$ was used as gas phase (Table 1). Addition of cysteine to preincubated homogenates also gave slight protection of enzyme (Table 1).

*In the presence of substrate.* Evidence of inactivation of acid phosphatase during incubation of liver homogenate with phenyl phosphate at 37° is presented in Fig. 4.

With 0·15 M-phenyl phosphate, and with the substrate as buffer (pH 5·9), 4 mg. of liver showed maximum enzyme activity during incubation for 10 min. and this activity was not influenced by the

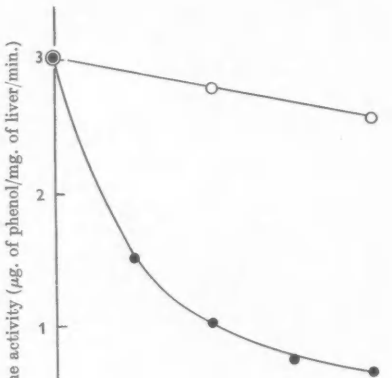


Fig. 2. Acid-phosphatase activities of 0·6% liver homogenate (containing 0·01 M-sodium malonate–HCl buffer, pH 5·9) when kept in an ice bath (○), and when incubated, with shaking, at 37° (●). At specified times, samples (0·5 ml.) of homogenate were used for enzyme assays. Assay conditions: 0·10 M-sodium phenyl phosphate, pH 5·9; total vol., 5·5 ml.; incubation for 10 min. at 37°.

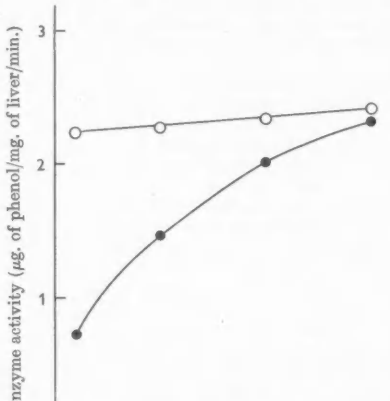


Fig. 3. Effects of sodium malonate–HCl buffer (pH 5·9) on inactivation of acid phosphatase in preincubated liver homogenate. Samples (20 ml.) of 0·6% liver homogenate, containing malonate buffer as indicated, were incubated in stoppered 50 ml. conical flasks for 1 hr. at 37° (with shaking) and portions (0·5 ml.) used for enzyme assays. Conditions of enzyme assay: 0·15 M-sodium phenyl phosphate, pH 5·9; total vol., 5·5 ml.; incubation for 10 min. at 37°. ○, Activities of fresh homogenates; ●, activities after preincubation for 1 hr.

Table 1. *Acid-phosphatase activities of mouse-liver homogenate preincubated with various buffer systems*

Samples (20 ml.) of 0·6% liver homogenate, containing indicated buffers (pH 5·9), were incubated, with shaking, in stoppered 50 ml. conical flasks (or Thunberg tubes when nitrogen was used as gas phase) for 1 hr. at 37° and portions (0·5 ml.) were used for enzyme assays. Residual enzyme activities are expressed as percentages of the activity of fresh 0·6% homogenate containing 0·10M-sodium malonate–HCl buffer (pH 5·9). Conditions of enzyme assay: 0·15M-sodium phenyl phosphate, pH 5·9; total vol., 5·5 ml.; incubation for 10 min. at 37°.

| Preincubated homogenate | | | Residual enzyme activity (%) |
|---|---|---|---|
| Buffer system (0·10M) | Gas phase | Cysteine (0·015M) | |
| Sodium malonate–HCl | Air | – | 88 |
| Sodium citrate–HCl | Air | – | 77 |
| Disodium EDTA–NaOH | Air | – | 60 |
| Sodium succinate–HCl | Air | – | 47 |
| Sodium acetate–HCl | Air | – | 23 |
| Sodium acetate–HCl | Air | + | 33 |
| Sodium acetate–HCl | Nitrogen | – | 33 |
| Sodium acetate–HCl | Nitrogen | + | 49 |

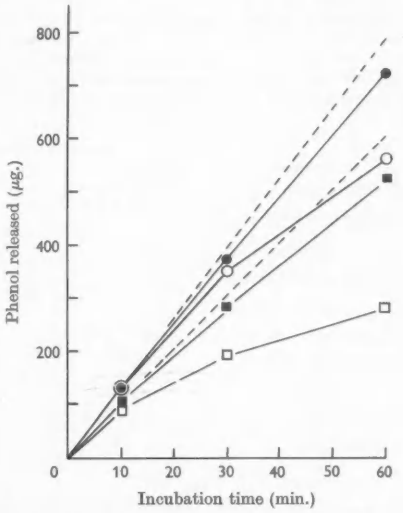


Fig. 4. Time course of hydrolysis of phenyl phosphate by mouse-liver acid phosphatase. Reaction mixtures (5·5 ml.; pH 5·9) contained 4 mg. of liver and: ○, 0·15M-sodium phenyl phosphate; ●, 0·15M-sodium phenyl phosphate + 0·05M-sodium malonate; □, 0·04M-sodium phenyl phosphate; ■, 0·04M-sodium phenyl phosphate + 0·05M-sodium malonate. Mixtures were incubated, with shaking, at 37°. Each point is the mean value of duplicate tubes. Broken lines are straight-line extrapolations of the maximum initial rates of hydrolysis obtained with the two concentrations of substrate.

Table 2. *Effects of ethylenediaminetetra-acetic acid and metal ions on mouse-liver acid-phosphatase activity*

Conditions of enzyme assay: 7 mg. of liver; 0·10M-sodium phenyl phosphate, pH 5·9 (adjusted with acetic acid); additions as shown in Table; total vol., 5·5 ml.; incubation for 6 min. at 37°. Enzyme activity was arrested by addition of 1·0 ml. of N-NaOH (0·325M with respect to tetrasodium salt of EDTA).

| Addition | Concn. (mM) | Percentage inhibition of acid-phosphatase activity |
|---|---|---|
| Sodium EDTA (pH 5·9) | 50 | 0 |
| Magnesium acetate | 10 | 0 |
| Calcium acetate | 10 | 0 |
| Barium chloride | 10 | 0 |
| Manganese chloride | 0·1 | 0 |
| Stannous chloride | 0·1 | 10 |
| Aluminium chloride | 1·0 | 45 |
| | 0·1 | 10 |
| Zinc sulphate | 1·0 | 75 |
| | 0·1 | 15 |
| Ferric sulphate | 0·1 | 50 |
| Lead nitrate | 0·1 | 0 |
| Silver nitrate | 0·1 | 65 |
| Copper sulphate | 0·1 | 85 |
| Mercuric acetate | 0·1 | 90 |

presence of malonate (0·05M). However, when the incubation period was increased to 60 min., the presence of malonate resulted in greater hydrolysis of phenyl phosphate over this period. Similar patterns of enzyme activity were obtained with 0·04M-phenyl phosphate and prolonged incubation, though enzyme inactivation in the absence of malonate, and degree of malonate protection, were obviously greater than when 0·15M-phenyl phosphate was used. With 0·04M-phenyl phosphate, significant enzyme inactivation occurred during incubation for 10 min. Measurements of the pH of the reaction mixtures, before and after incubation at 37°, showed that losses of activity in the absence of malonate buffer were not due to inadequate buffering by the substrate.

It was concluded that the mechanism of enzyme inactivation is the same as that previously observed in preincubated homogenates (Fig. 2) and that although phenyl phosphate has a protective

Table 3. *Effects of some added compounds on inhibition of mouse-liver acid phosphatase by $Fe^{3+}$, $Cu^{2+}$ and $Hg^{2+}$ ions*

Conditions of enzyme assay: 4 mg. of liver; 0·10 M-sodium phenyl phosphate, pH 5·9; inhibitory metal salts, 0·1 mM; added compounds (pH 5·9) as shown in Table; total vol., 5·5 ml.; incubation for 10 min. at 37°. Inhibitions are expressed as percentages of enzyme activities in the absence of inhibitory metal ions.

| Added compound | Concn. (M) | Enzyme inhibition (%) In the presence of ferric chloride | In the presence of copper sulphate | In the presence of mercuric acetate |
|---|---|---|---|---|
| None | — | 44 | 89 | 95 |
| Sodium malonate | 0·05 | 9 | 53 | 60 |
| Sodium citrate | 0·05 | 8 | — | — |
| Sodium EDTA | 0·05 | 29 | 3 | 43 |
| L-Cysteine | 0·003 | 0 | 0 | 3 |

action on liver acid phosphatase, the presence of another protective system, such as malonate, is required when incubation periods longer than 10 min., or substrate concentrations less than 0·1 M, are used.

### *Effects of ethylenediaminetetra-acetic acid and metal ions*

The results of studies of the effects of EDTA and selected metal ions on liver acid-phosphatase activity are presented in Table 2. In these experiments, pH adjustments of substrate and disodium EDTA were made with acetic acid and enzyme action was arrested by addition of 1 ml. of N-NaOH containing tetrasodium EDTA (0·325 M). This was added to the NaOH to dissolve metal hydroxides and thereby reduce their interference in spectrophotometric determinations of phenol. As in previous experiments, phenol values were confirmed by measurements of the inorganic phosphate released by enzyme action on phenyl phosphate.

EDTA was not inhibitory and none of the metal ions tested stimulated phenyl phosphate hydrolysis. It therefore appears that metal ions are not required for enzyme activity. This is consistent with the previous observation of linearity between acid-phosphatase activity and quantity of liver in the absence of added metal ions.

Marked inhibition of phenyl phosphate hydrolysis was obtained with $Hg^{2+}$, $Cu^{2+}$, $Ag^{+}$ and $Fe^{3+}$ ions (0·1 mM). Inhibition by $Fe^{3+}$ ions was of special interest as it raised the possibility that enzyme inactivation in preincubated homogenates (Fig. 2), and during incubation with substrate (Fig. 4), was due to $Fe^{3+}$ ions contained in the homogenate. To test this possibility, comparisons were made of the effects of malonate, citrate and EDTA on enzyme inhibitions by $Fe^{3+}$, $Hg^{2+}$ and $Cu^{2+}$ ions (Table 3). The efficiencies of these organic compounds in preventing enzyme inhibition by $Fe^{3+}$ ions closely paralleled their efficiencies in preventing enzyme inactivation in preincubated homogenates. Thus malonate and citrate afforded almost complete protection against $Fe^{3+}$ ions, whereas EDTA gave only slight protection. By contrast, EDTA was more efficient than malonate in protecting liver acid phosphatase against inhibitions by $Hg^{2+}$ and $Cu^{2+}$ ions. Addition of cysteine to liver homogenate gave complete protection against $Fe^{3+}$, $Hg^{2+}$ and $Cu^{2+}$ ions (Table 3) and this provided evidence that the inhibitory actions of these metal ions were due to reaction with sulphydryl groups.

Table 4. *Effects of some sulphydryl inhibitors on mouse-liver acid-phosphatase activity*

Conditions of enzyme assay: 4 mg. of liver; 0·10 M-sodium phenyl phosphate, pH 5·9; additions as shown in Table; total vol., 5·5 ml.; incubation for 10 min. at 37°. Inhibitions are expressed as percentages of enzyme activities obtained in the absence of sulphydryl inhibitors.

| Additions | Concn. (mM) | Enzyme inhibition (%) |
|---|---|---|
| *p*-Chloromercuribenzoate | 0·1 | 61 |
| *p*-Chloromercuribenzoate + L-cysteine | 0·1 + 3·0 | 0 |
| *p*-Chloromercuribenzoate + sodium malonate (pH 5·9) | 0·1 + 50 | 57 |
| Phenyl mercuric acetate | 0·1 | 61 |
| Phenyl mercuric acetate + L-cysteine | 0·1 + 3·0 | 6 |
| *o*-Iodosobenzoate | 0·1 | 17 |
| *o*-Iodosobenzoate + L-cysteine | 0·1 + 3·0 | 4 |

### *Effects of some typical sulphydryl inhibitors*

In view of the sensitivity of liver acid phosphatase to traces of $Hg^{2+}$, $Cu^{2+}$ and $Fe^{3+}$ ions (Table 2), studies were made of the effects of some other typical SH inhibitors. *p*-Chloromercuri-

benzoate, phenyl mercuric acetate and *o*-iodosobenzoate inhibited acid-phosphatase activity and these inhibitions were prevented by the presence of cysteine (Table 4). Malonate had no significant effect on enzyme inhibition by *p*-chloromercuribenzoate (Table 4).

*Effects of inorganic phosphate and other anions*

Previous workers have reported that certain anions, e.g. oxalate (Belfanti, Contardi & Ercoli, 1935), fluoride (Belfanti *et al.* 1935; Goodlad & Mills, 1957) and tartrate (Goodlad & Mills, 1957), inhibit liver acid-phosphatase activity. The effects of a number of different anions were tested under the assay conditions used in the present work (Table 5). Under these conditions fluoride and arsenate (0·01 M) acted as strong inhibitors (approx. 60 % inhibition). Phosphate, pyrophosphate and cyanide caused significant inhibition (20–30 %), whereas citrate, β-glycerophosphate, oxalate and tartrate had little effect. Determinations of enzyme activities in the presence of phosphate and pyrophosphate were based solely on measurements of phenol released.

Table 5. *Effects of some anions on mouse-liver acid-phosphatase activity*

Conditions of enzyme assay: 4 mg. of liver; 0·1 M-sodium phenyl phosphate, pH 5·9; additions (previously adjusted to pH 5·9 with HCl) as shown in Table; total vol., 5·5 ml.; incubation for 10 min. at 37°.

| Addition (0·01 M) | Enzyme inhibition (%) |
|---|---|
| Sodium fluoride | 66 |
| Sodium arsenate | 58 |
| Sodium phosphate | 29 |
| Sodium cyanide | 26 |
| Potassium pyrophosphate | 20 |
| Sodium citrate | 12 |
| Sodium β-glycerophosphate | 7 |
| Potassium oxalate | 7 |
| Sodium L-tartrate | 6 |

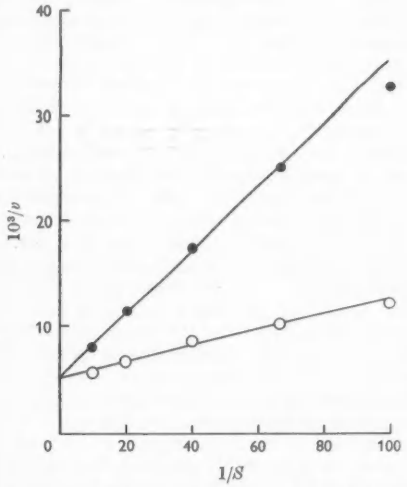


Fig. 5. Double-reciprocal plots (Lineweaver & Burk, 1934) of hydrolysis of phenyl phosphate by mouse-liver acid phosphatase acting in the absence (○) and the presence (●) of 0·01 M-sodium phosphate. Reaction mixtures (5·5 ml.) contained 4 mg. of liver and 0·05 M-sodium malonate and were incubated at pH 5·9 for 10 min. at 37°. Values for reaction velocity ($v$) are expressed as μg. of phenol/10 min.

Inhibition by inorganic phosphate was competitive. This is illustrated in Fig. 5, in which experimental results have been recorded as double-reciprocal plots (Lineweaver & Burk, 1934). In the experiments of Fig. 5 the enzyme reaction mixtures also contained 0·05 M-malonate (pH 5·9). This ensured reliable values for enzyme activities over the substrate concentration range used (0·01–0·10 M) and eliminated the complicating factor of substrate protection of enzyme. Even in the presence of malonate, substrate concentrations of approximately 0·1 M were required for enzyme saturation. The Michaelis constant, calculated from Fig. 5, was 13 mM.

*Comparison of liver acid-phosphatase activities of well-fed and of starved mice*

To determine whether starvation affected liver acid-phosphatase activities and thus accounted for the lower values obtained by Tsuboi (1952), measurements were made of the liver-enzyme activities of mice starved for 22–28 hr. Mean values [± S.D. (no. of animals)] of 3·5 ± 0·5 (10) μg. of phenol/mg. of liver/min. and 102 ± 14 (10) μg. of phenol/mg. of liver nitrogen/min. were obtained. Corresponding values for well-fed mice were 3·4 ± 0·4 (9) μg. of phenol/mg. of liver/min. and 91 ± 11 (9) μg. of phenol/mg. of liver nitrogen/min.

## DISCUSSION

The association of liver acid phosphatase with a special class of cytoplasmic granules, the lysosomes, has already been clearly demonstrated by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) in experiments with rat liver and with β-glycerophosphate as substrate. It follows that, to obtain maximum values for liver acid-phosphatase activity, the selected method must ensure (*a*) complete liberation of active enzyme from intracellular particles, (*b*) prevention of enzyme inactivation in the preparation of liver homogenates, and (*c*) prevention of enzyme inactivation during incubation of homogenate sample with substrate. The

described method appears to satisfy these requirements and has led to higher values for mouse-liver acid-phosphatase activity than have been reported by previous workers (Kochakian & Fox, 1944; Tsuboi, 1952). By virtue of this high enzyme activity, and the permissible dilution of liver homogenates for enzyme assays, the spectrophotometric procedure of Brawerman & Chargaff (1954) has been adopted for measurement of phenyl phosphate hydrolysis by liver homogenates.

In view of the sensitivity of liver acid phosphatase to $Hg^{2+}$, $Cu^{2+}$, $Ag^{+}$ and $Fe^{3+}$ ions (Table 2) and to *p*-chloromercuribenzoate, phenyl mercuric acetate and *o*-iodosobenzoate (Table 4), it is concluded that liver acid phosphatase is a sulphydryl enzyme. In studies of acid phosphatases of other mammalian tissues, namely erythrocytes and prostate, Abul-Fadl & King (1949) obtained evidence that these enzymes are sulphydryl enzymes and this conclusion has been confirmed by later studies of purified enzymes from these tissues (Tsuboi & Hudson, 1953, 1955).

The apparent dependence of liver acid phosphatase upon sulphydryl groups may explain the enzyme inactivation which occurs in liver homogenate incubated at 37° either alone (Fig. 2) or with phenyl phosphate (Fig. 4). The possibility that such inactivation is due to oxidation of sulphydryl groups is supported by the higher enzyme activity shown by liver homogenate preincubated with nitrogen as gas phase and in the presence of cysteine (Table 1). Acid-phosphatase inactivation by proteolytic enzymes contained in homogenates appears unlikely in view of the short incubation periods (1 hr.) used in inactivation studies and in view of the protective actions of malonate and citrate (Figs. 3 and 4, and Table 1).

The technique of using substrate as buffer (pH 5·9) has permitted assessment of the effects of added buffer systems on phenyl phosphate hydrolysis by liver acid phosphatase. Of the various buffers tested (malonate, citrate, EDTA, succinate, acetate), malonate ($pK_2$ 5·68; Umbreit, Burris & Stauffer, 1951) appears to be the most suitable in that, although without obvious effect on the enzyme reaction by itself, it provides greatest protection of acid phosphatase in liver homogenate incubated at 37°. Citrate is almost as efficient as malonate in preventing enzyme inactivation but has the undesirable property of causing enzyme inhibition during assays, particularly when low substrate concentrations are used.

Studies of the effects produced by malonate, citrate and EDTA have revealed close parallels in the efficiencies of these compounds in preventing enzyme inactivation in preincubated homogenate (Table 1) and in preventing enzyme inhibition by added $Fe^{3+}$ ions. These results suggest that traces of $Fe^{3+}$ ions in liver homogenates are responsible for the observed enzyme inactivations and that enzyme protection by malonate and citrate is due to conversion of $Fe^{3+}$ ions into non-inhibitory metal complexes (see Martell & Calvin, 1952). Inhibition of acid phosphatases of erythrocytes and prostate by added $Fe^{3+}$ ions, in the presence of acetate buffer, pH 5·0, has been observed by Abul-Fadl & King (1949) and these authors have also shown that such inhibition is prevented by citrate buffer of the same pH. However, the lack of suitable methods for determining $Fe^{3+}$ ion contents of liver homogenates at pH 5·9 has prevented further studies of the possible inactivation of liver acid phosphatase by such ions.

In these experiments, enzyme inactivation has also been prevented by phenyl phosphate concentrations of 0·1 M or greater. The protection of liver acid phosphatase by phenyl phosphate has been shown to vary with the concentration of phenyl phosphate used (Fig. 4). The mechanism of phenyl phosphate protection is uncertain. Possible mechanisms are (*a*) union between phenyl phosphate and sulphydryl groups of acid phosphatase and consequent prevention of their oxidation and (*b*) combination of phenyl phosphate with inactivating agent and prevention of enzyme inactivation by this agent.

Another important feature of phenyl phosphate concentration is that approximately 0·1 M-solutions are required for enzyme saturation with substrate. A similar observation has been made by Tsuboi (1952). This concentration is much higher than that (5 mM) used by Robinson *et al.* (1939) and by the majority of workers with liver acid phosphatase.

The optimum pH for hydrolysis of phenyl phosphate by mouse-liver acid phosphatase is shown to be pH 5·9–6·1 (Fig. 1). This pH is higher than that (pH 4·9) used by Robinson *et al.* (1939) and by workers with liver acid phosphatase (e.g. pH 5·5, Tsuboi, 1952; pH 5·0, Abul-Fadl & King, 1949; pH 4·9, Kochakian & Fox, 1944; pH 4·6, Greenstein, 1942), but is almost identical with that reported by Schønheyder (1952) for seminal acid-phosphatase activity with phenyl phosphate. The possibility that the rate of inactivation of liver acid phosphatase varies with pH of incubation has not been examined in the present studies.

Inorganic phosphate acts as a competitive inhibitor of liver acid phosphatase. Inorganic phosphate behaves similarly in the hydrolysis of phenyl phosphate by seminal acid phosphatase (Schønheyder, 1952). This property of inorganic phosphate, considered together with the non-specificity of acid phosphatases, and the demonstration by Cohn (1949) of enzymic cleavage of P–O bonds, indicates that the union between substrate and acid phosphatase involves the phos-

phate moieties of phosphomonoester substrates. The failure of β-glycerophosphate to inhibit phenyl phosphate hydrolysis (Table 5) suggests that β-glycerophosphate has little affinity for the enzyme under the conditions used. This effect may be due to differences in the dissociations of the phosphate moieties of phenyl phosphate and β-glycerophosphate at pH 5·9 (Delory, Wiberg & Hetherington, 1955). Arsenate and pyrophosphate produce significant inhibition of phenyl phosphate hydrolysis (Table 5) and these inhibitions are presumed to be similar to that caused by inorganic phosphate. Fluoride and cyanide also inhibit liver acid phosphatase (Table 5), but, at present, no satisfactory explanations of these inhibitions can be given. Citrate, L-tartrate and oxalate resemble β-glycerophosphate in producing little enzyme inhibition under conditions suitable for maximum rates of hydrolysis of phenyl phosphate (Table 5).

The described method of measuring liver acid-phosphatase activity is simple and rapid and should prove useful as an assay method in the isolation of liver acid phosphatase. Awareness of the instability of this enzyme and of the prevention of enzyme inactivation by malonate should assist in obtaining high yields of the enzyme in isolation procedures.

## SUMMARY

1. An examination has been made of the effects of variations in assay conditions on the rate of hydrolysis of phenyl phosphate by mouse-liver acid phosphatase at 37°.

2. Maximum rates of hydrolysis were obtained with 0·1M-phenyl phosphate (pH 5·9–6·1) and incubation periods of 5–10 min. There appeared to be no requirement for added metal ions.

3. Inactivation of acid phosphatase, shown to occur in liver homogenates incubated at pH 5·9 in both the absence and the presence of phenyl phosphate, was prevented by malonate. Evidence suggesting that enzyme inactivation is caused by traces of $Fe^{3+}$ ions in liver homogenates is presented.

4. Hydrolysis of phenyl phosphate was inhibited by $Hg^{2+}$, $Cu^{2+}$ and $Fe^{3+}$ ions, *p*-chloromercuribenzoate, phenyl mercuric acetate and *o*-iodosobenzoate, and these inhibitions were prevented by cysteine. This suggests that liver acid phosphatase is a sulphydryl enzyme.

5. Under optimum conditions of phenyl phosphate hydrolysis, fluoride, arsenate, phosphate, cyanide and pyrophosphate caused significant enzyme inhibition, whereas citrate, β-glycerophosphate, oxalate and L-tartrate had little effect. Inhibition by phosphate is shown to be competitive.

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# Glucose Utilization in Sheep

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In ruminants only small quantities of glucose are absorbed from the alimentary tract, most of the dietary energy being supplied by short-chain volatile fatty acids produced by the fermentation of ingested carbohydrates in the rumen. In addition, blood glucose concentrations in ruminants are considerably lower than those of non-ruminants, and ruminants are relatively insensitive to insulin. These major differences in carbohydrate metabolism have led to considerable speculation on the role of glucose in ruminant metabolism (Lindsay, 1959), but in the present studies measurements of glucose-utilization rates in sheep have demonstrated that glucose is quantitatively only slightly less important in this species than in non-ruminants.

Isotope-dilution techniques, which provide the only satisfactory methods for the measurement of turnover rates in the intact animal, have been used to determine glucose-utilization rates in rats (Feller, Strisower & Chaikoff, 1950; Baker, Shipley, Clark & Incefy, 1959), man (Baker, Shreeve, Shipley, Incefy & Miller: 1954) and dogs (Searle, Strisower & Chaikoff, 1954; Steele, Wall, de Bodo & Altszuler, 1956). Studies of ruminants, however, have been limited to those of Baxter, Kleiber & Black (1955) and Kronfeld, Tombropoulos & Kleiber (1959), who used single-injection procedures to measure glucose-turnover rates in cows. In the present investigation glucose-utilization rates in fed and starved sheep have been determined by isotope dilution with the constant-infusion method of Steele *et al.* (1956). The general procedure employed was similar to that described earlier for the measurement of acetate-utilization rates in sheep (Annison & Lindsay, 1961). Estimates of glucose-pool size and glucose 'space' (the volume of distribution of the glucose pool) were also obtained. In some experiments these parameters of glucose metabolism were measured by isotope dilution with a single-injection procedure, but utilization rates determined by this method were significantly higher than those obtained by constant infusion of $^{14}$C-labelled glucose.

The effects of glucose load on the entry of endogenous glucose were examined by measuring utilization rates at high concentrations of plasma glucose. Entry of glucose appeared to be markedly inhibited under these conditions.

## MATERIALS AND METHODS

*Experimental animals.* Twelve Merino ewes, age 2–3 years, were randomly divided into two groups of six animals, each of which was fed on one of the following diets: 400 g. of lucerne chaff (diet 1); 400 g. of lucerne chaff and 500 g. of maize (diet 2). The animals, which were housed indoors, were fed once daily and trained to eat their food within 2 hr. After transfer to new diets animals were allowed a period of 2–3 months to adjust to the new feeding régime before being used for experiments. All animals fed with diet 1 lost weight at an average rate of 0·2 kg./week during this time. This loss of weight continued at approximately the same rate for a further 3–4 months in half of the group, the remainder reaching equilibrium during this period. The second group (diet 2) gained weight at about 0·3 kg./week in the adjustment period, but subsequently showed variable weight changes which did not exceed 0·5 kg./week.

To accustom the animals to the conditions of the experiments they were restrained in the cage used for the infusion experiments for several hours each day over a period of 3–4 weeks. Experiments on fed animals were started 2 hr. after the end of the feeding period, and starved animals were not fed on the day of the experiment.

*Experimental procedure.* The general procedure described earlier (Annison & Lindsay, 1961) was used, with some modifications. Constant infusion (0·3 ml./min.) of labelled glucose was maintained with a continuous-injection apparatus (Palmer Instruments Ltd., London). Since utilization rates can only be measured under steady-state conditions, i.e. when the plasma glucose concentrations remain reasonably constant, care was taken to minimize disturbance of the animals before and during experiments. For this reason the polyethylene catheters were inserted into the jugular veins on the day before an experiment. Experiments were rejected if the plasma glucose concentration varied by more than $\pm 10\%$ of the mean value observed during the sampling period.

*Radioactive glucose.* Uniformly labelled D-[$^{14}$C]glucose was obtained from The Radiochemical Centre, Amersham, Bucks. Aqueous solutions of labelled glucose were prepared (50 $\mu$c/mg./ml.) and stored at $-10°$ until diluted in 0·1% glucose immediately before use. The radioactivity of each batch of labelled glucose was checked by wet oxidation of a sample to barium carbonate.

### *Chemical determinations*

*Determination of plasma glucose.* The erythrocytes of mature sheep usually contain little or no glucose (Reid, 1953) and examination of the animals available to us in this laboratory indicated that the ratio plasma glucose concentration : erythrocyte glucose concentration was in most

cases at least 20:1. Erythrocyte glucose concentrations were calculated from the values obtained for whole blood and plasma, and from the haematocrit value. There is considerable uncertainty about correction factors for trapped plasma in haematocrit determinations in sheep (Turner & Hodgetts, 1959) and it is correspondingly difficult to decide whether or not glucose is present in the erythrocyte in low concentration or is absent. For this reason the experimental work was based on plasma glucose. This procedure also avoided possible variations in blood glucose concentration caused by the rapid release of erythrocytes from the spleen after even slight disturbance of the animal (Turner & Hodgetts, 1959). The specific glucose-oxidase procedure of Huggett & Nixon (1956) was used to estimate plasma glucose concentrations. Heparin was used as anticoagulant, and sodium fluoride (25 mg.) was added to each 10 ml. of blood to inhibit glycolysis.

*Assay of labelled glucose in plasma.* Two methods were used to determine the specific activity of plasma glucose. In the early part of the investigation, when only an end-window Geiger–Müller tube was available for radioactivity measurements, the periodate-oxidation procedure of Dunn, Friedmann, Maas, Reichard & Weinhouse (1957) was used. When more sensitive radioactive counting facilities became available (gas-flow counter) glucose was isolated from plasma as the osazone, oxidized to $CO_2$ by the method of Van Slyke & Folch (1940) and counted as barium carbonate. The two methods gave closely similar results when used to assay radioactive glucose of known specific activity contained in aqueous solution or in plasma. Although the periodate-oxidation procedure is more time-consuming and less specific than the osazone method, an important advantage of the technique is its greater sensitivity. This is in consequence of the much smaller amounts of added carrier glucose necessary in the periodate method, and from the inevitable dilution of glucose carbon with carbon from phenylhydrazine in the osazone derivative. The periodate-oxidation assay thus permits the use of lower concentrations of [$^{14}$C]glucose in the infusion experiments, which results in an important saving in costs when experiments with large animals are involved.

(i) Periodate oxidation of plasma glucose. The method of Dunn *et al.* (1957) was used, with some modifications. Carrier glucose (4 mg.) was added to plasma (5 ml.), which was diluted with water (5 ml.) before deproteinization with 0·3 N-barium hydroxide (7·5 ml.) and 0·3 N-zinc sulphate (7·5 ml.). Portions of the filtrate (10 ml.) were oxidized in an all-glass apparatus. Aeration of the acidified solution at room temperature to remove $CO_2$ before the oxidation of formate was found to be inadequate, but satisfactory results were obtained when filtrates were aerated at 90–95° for 15 min. Recoveries of $CO_2$ by this method were 95–100% of the theoretical yield. The periodate-oxidation method is not specific for glucose, a possible major source of error in some circumstances being the decarboxylation of aliphatic hydroxy and keto acids by mercuric oxide during the conversion of formic acid into $CO_2$. To avoid errors from this source, samples of plasma obtained during each experiment were assayed with the omission of the periodate-oxidation stage, and the results used to correct those obtained after the complete oxidation procedure. Control experiments established that $^{14}CO_2$ was not obtained from labelled glucose if the periodate-oxidation step was omitted.

(ii) Isolation and oxidation of plasma glucose as osazone. Carrier glucose (20 mg.) was added to plasma (5 ml.), which was deproteinized by pouring into ethanol (20 ml.). After removal of precipitated protein, the filtrate was concentrated, by heating at 50–60° in a stream of air, to a volume of 4–5 ml. and the glucosazone prepared. The osazones were recrystallized twice from the system pyridine–water (1:4, v/v).

The osazones (2–3 mg.) were oxidized by the procedure of Van Slyke & Folch (1940), the liberated $CO_2$ being absorbed in 2 ml. of $CO_2$-free 0·5 N-sodium hydroxide. After the addition of 1 ml. of 5% ammonium′ chloride, 0·2 ml. of 10% barium chloride was added and the precipitated barium carbonate isolated and assayed for radioactivity.

*Assay of specific activity of blood carbon dioxide.* The method described by Annison & Lindsay (1961) was used.

*Assay of radioactive barium carbonate.* Radioactive barium carbonate was collected by filtration on Whatman no. 542 paper circles with an apparatus which eliminated the transfer of papers and precipitates to separate planchets. The paper circles were held on perforated-brass planchets by a stainless-steel Gooch-type funnel and the planchets then screwed directly on to filter stems. Weights of barium carbonate in the range 10–25 mg. were usually employed, but satisfactorily reproducible results were obtained down to weights of 5 mg. (2 mg./cm.$^2$). The barium carbonate samples were assayed for radioactivity with an end-window Geiger–Müller tube or with a gas-flow counter fitted with a Micromil window (Nuclear-Chicago Corp., model D-47). Counts were corrected for self-absorption by the procedure of Hendler (1959), in which results are corrected to a standard weight of barium carbonate (15 mg.). Calibrated samples of sodium [$^{14}$C]carbonate and barium [$^{14}$C]carbonate obtained from Tracerlab Inc., U.S.A., were used as absolute standards.

*Measurement of utilization rate.* The term utilization, as used in this paper, has been defined earlier (Annison & Lindsay, 1961). In the studies with acetate (Annison & Lindsay, 1961) it was found unnecessary to use a priming dose of isotope (Steele *et al.* 1956) since the continuous infusion of labelled acetate gave rise to constant specific activities of plasma acetate after 30–60 min. With glucose, however, steadily increasing specific activities of plasma

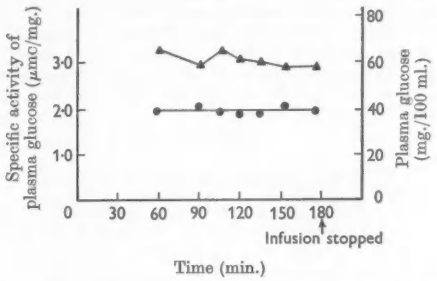


Fig. 1. Plasma glucose specific activity in a typical experiment on a starved sheep. Priming dose, 15·4 μc; infusion rate 0·17 μc/min. ●, Specific activity of plasma glucose; ▲, plasma glucose concentration.

Table 1. *Glucose-utilization rates in starved sheep measured by infusion and single-injection (S.I.) techniques*

| Sheep no. | Date | Diet | Technique | Average concn. of plasma glucose (mg./100 ml.) | Glucose space (% of body wt.) | Glucose utilization (mg./min./kg.) |
|---|---|---|---|---|---|---|
| 47 | 10. ix. 59 | 2 | S.I. | 85 | 20 | 2·2 |
| 47 | 24. ix. 59 | 2 | S.I. | 85 | 19 | 2·1 |
| 8816 | 12. iv. 60 | 2 | S.I. | 75 | 17 | 3·1 |
| 9397 | 12. x. 60 | 1 | S.I. | 70 | 16 | 2·5 |
| 10 | 13. x. 60 | 1 | S.I. | 71 | 18 | 2·8 |
| 58 | 13. x. 60 | 1 | S.I. | 72 | 16 | 3·1 |
| 47 | 22. ii. 60 | 1 | Infusion | 60 | 23 | 1·6 |
| 8816 | 24. ii. 60 | 2 | Infusion | 70 | 17 | 1·4 |
| 8816 | 2. iii. 60 | 2 | Infusion | 50 | 22 | 1·2 |
| 8816 | 10. iii. 60 | 2 | Infusion | 60 | 22 | 1·8 |
| 8816 | 16. ix. 60 | 1 | Infusion | 48 | 19 | 1·2 |
| 9397 | 25. x. 60 | 1 | Infusion | 73 | 21 | 1·5 |
| 10 | 18. viii. 60 | 2 | Infusion | 56 | 25 | 1·6 |
| 58 | 8. iii. 60 | 1 | Infusion | 41 | 24 | 1·1 |
| 58 | 18. x. 60 | 1 | Infusion | 75 | 22 | 1·1 |
| 51 | 11. xi. 59 | * | Infusion | 54 | 25 | 1·8 |
| 51 | 26. ii. 60 | 2 | Infusion | 73 | 21 | 1·7 |
| 51 | 4. iii. 60 | 2 | Infusion | 45 | 27 | 1·4 |
| 9 | 6. vii. 60 | 2 | Infusion | 57 | 30 | 1·8 |

* Diet: 400 g. of lucerne and 250 g. of maize/day.

glucose were obtained during the infusion of labelled glucose, as expected from the results of Steele *et al.* (1956) with dogs. When a priming dose was used, however, and specific activities of plasma glucose were plotted against time, in most experiments a straight-line plot was observed over the period 90–150 min. after the start of the infusion. This line was usually horizontal (Fig. 1), but occasionally showed a slight positive or negative slope. It was found that a priming dose of 15 $\mu$c of labelled glucose followed by the infusion of labelled isotope at 0·17 $\mu$c/min. was suitable.

In the single-injection experiments, blood samples were taken at 5 min. intervals after the injection of 50 or 100 $\mu$c of labelled glucose, and the specific activity of plasma glucose was plotted against time on a semi-logarithmic scale. In the calculation of utilization rate first-order reaction kinetics were assumed to apply (Dunn *et al.* 1957).

Glucose-utilization rates were calculated as mg./min./kg. body wt. (mg./min./kg.). A factor to be borne in mind when utilization rates measured in different species are compared on the basis of body weight is that in sheep a relatively larger proportion of body weight is represented by the alimentary tract than in some non-ruminants, e.g. dog.

## RESULTS

### *Glucose utilization in starved sheep*

The results obtained with the constant-infusion and single-injection techniques in starved (24 hr.) sheep are summarized in Table 1. Significantly higher results were given by the single-injection method, the mean value of six experiments being 2·9 ± 0·18 (S.E.) mg./min./kg. (range 2·1–3·1). Corresponding values for utilization rate by the continuous-infusion techniques showed a mean for 13 experiments of 1·5 ± 0·07 mg./min./kg. (range 1·1–1·8). It must be noted that average plasma glucose values were somewhat higher in the single-injection experiments than those observed in the continuous-infusion studies (Table 1), but since similar experimental techniques were used in both series of experiments these differences were presumably fortuitous. Some correlation between utilization rate and plasma glucose concentration was observed (Fig. 4) but a comparison of utilization rates measured in the same animals (sheep 47, 8816 and 58, Table 1) by both techniques suggests that the higher values obtained by single injection cannot be fully accounted for by differences in plasma glucose concentration. Lower values for glucose space were obtained by single injection (mean 18 %, range 16–20 %) than by constant infusion (mean 23 %, range 17–30 %).

Table 2. *A comparison of results obtained for sheep in the present studies with those for starved dogs (Steele, Wall, de Bodo & Altszuler, 1956)*

| Subject | No. of experiments | Mean value and range of body wt. ($W$) (kg.) | Mean value and range of utilization rate (g./hr./$W^{0.6}$) |
|---|---|---|---|
| Fed sheep | 7 | 36·6 (31·4–44·0) | 0·68 (0·51–0·91) |
| Starved sheep | 13 | 33·5 (24·8–41·5) | 0·37 (0·26–0·48) |
| Starved dogs | 10 | 19·1 (13·8–24·1) | 0·49 (0·42–0·67) |

When calculated in terms of metabolic body weight (Brody, 1945), utilization rates in starved sheep were about 75% of those obtained from infusion experiments on starved dogs (Steele *et al.* 1956), as shown in Table 2.

There was no obvious correlation between diet and glucose turnover in starved sheep (Table 1) although the total number of experiments was too small for adequate comparison in view of the wide range of values obtained on each diet.

*Glucose utilization in fed sheep*

Plasma glucose concentrations were found to be sufficiently constant in the period 2–5 hr. after the end of feeding to allow glucose utilization to be measured by constant infusion. The specific activity of plasma glucose reached a plateau value after about the same time interval (90–100 min.) as with starved sheep (Fig. 2). Considerably higher entry rates were observed after feeding (Table 3) with a mean value of $2{\cdot}6 \pm 0{\cdot}2(7)$ mg./min./kg. (range 2·1–3·4) and plasma glucose values were significantly higher than in starved (24 hr.) animals on similar diets (Table 1). Measurements, with one exception, were made on animals fed with the high-starch diet (2), which would be expected to yield high glucose-entry rates.

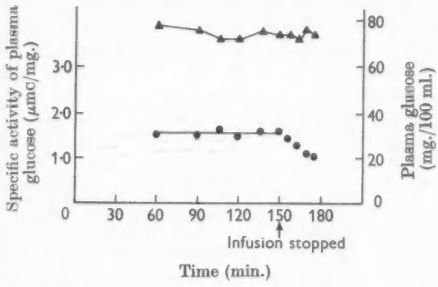


Fig. 2. Plasma glucose specific activity in a typical experiment on a fed sheep. Priming dose, 15·0 μc; infusion rate 0·17 μc/min. ●, Specific activity of plasma glucose; ▲, plasma glucose concentration.

*Effects of glucose load on utilization rate*: The constant-infusion technique allowed the plasma glucose concentration to be raised by including carrier glucose with labelled glucose in the infusion solution. The results of a typical experiment are shown in Fig. 3, and the results of five experiments on starved sheep are summarized in Table 4. At enhanced concentrations of plasma glucose the measured glucose-utilization rates were only slightly higher than, or identical with, the entry rate of unlabelled glucose. In addition, the specific activity of plasma glucose in the period after the cessation of infusion of glucose was largely unchanged (Fig. 3). A wide range of values for glucose space was obtained during these experiments (Table 4).

*Relationship of plasma glucose concentration to utilization rate.* When the utilization rates obtained in all continuous-infusion experiments on fed and starved sheep were plotted against the corresponding plasma glucose values (Fig. 4) some dependence of utilization rate on glucose concentration was clearly evident. A line corresponding to the equation $y = 0{\cdot}0273x + 0{\cdot}04$ was found to be significant ($P$ 0·01). This linear relationship was more apparent when the results obtained with any single animal were considered, and six values obtained with one sheep (8816) are also shown in Fig. 4.

*Glucose utilization on fasting.* Measurements were made on two sheep in the immediate post-feed period, and 24 and 48 hr. after feeding. A steady decline in plasma glucose value and utilization rate was observed in each case (Table 5).

*Specific activity of blood carbon dioxide.* Samples of venous blood were assayed at intervals during the infusion of labelled glucose and the results of a typical experiment are shown in Fig. 3. The specific activity of the $CO_2$ continued to rise throughout the infusion period.

A rough estimate of the contribution of glucose to overall oxidative metabolism was obtained by comparing the average specific activity of $CO_2$ with that of the specific activity of plasma glucose during the period of constant glucose specific

Table 3. *Glucose-utilization rates measured after feeding*

| Sheep no. | Date | Diet | Concn. of plasma glucose (mg./100 ml.) | Glucose space (% of body wt.) | Utilization rate (mg./min./kg.) |
|---|---|---|---|---|---|
| 8816 | 27. iv. 60 | 2 | 99 | 30 | 3·1 |
| 8816 | 29. iv. 60 | 2 | 77 | 29 | 2·4 |
| 51 | 16. iii. 60 | 2 | 79 | 30 | 3·4 |
| 9 | 5. vii. 60 | 2 | 99 | 19 | 2·2 |
| 9427 | 21. vi. 60 | 2 | 110 | 30 | 3·2 |
| 10 | 17. viii. 60 | 2 | 61 | 29 | 2·1 |
| 58 | 16. vi. 60 | 1 | 85 | 22 | 2·6 |

activity. In starved sheep the specific activity of blood $CO_2$ carbon was within the range 11–19% (five experiments) of that of plasma glucose carbon. Two experiments with fed animals gave values of 22 and 23%. In a further two experiments in which carrier glucose was infused with

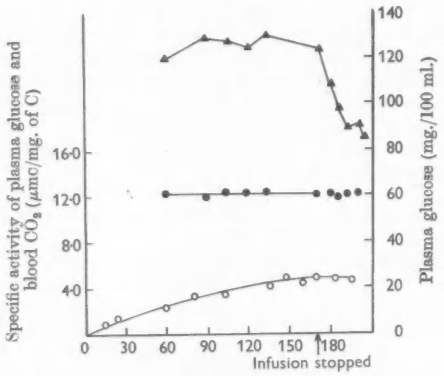


Fig. 3. Effects of glucose load on endogenous entry of glucose. Priming dose, 15·4 μc; infusion rate, 132 mg., 0·66 μc/min. Specific activity of infused glucose 5·0 μmc/mg.; average specific activity of plasma glucose, 5·0 μmc/mg. ●, Specific activity of plasma glucose; ▲, plasma glucose concentration; ○, specific activity of blood $CO_2$.

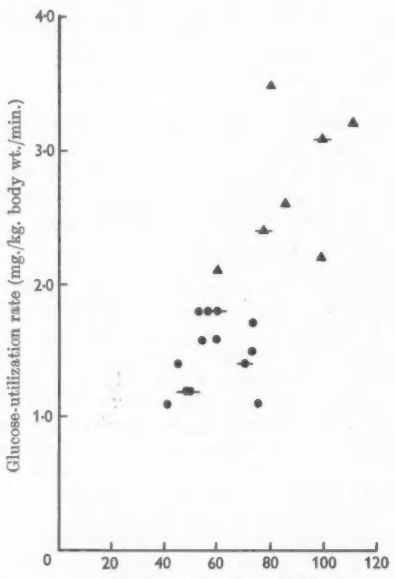


Fig. 4. Relationship between glucose-utilization rate and plasma glucose concentration, data obtained from all constant-infusion measurements on fed and starved sheep being used. ●, Starved sheep; ▲, fed sheep. Symbols with horizontal line (e.g. –●–) refer to sheep 8816.

Table 4. *Inhibition of entry of endogenous glucose at high concentrations of plasma glucose*

| Sheep no. | Concn. of plasma glucose (mg./100 ml.) | Glucose space (% of body wt.) | Rate of infusion of unlabelled glucose (*A*) (mg./min.) | Total entry rate of glucose (*B*) (mg./min.) | Utilization rate (mg./min./kg.) | Entry rate of endogenous glucose (*B* – *A*) (mg./min.) |
|---|---|---|---|---|---|---|
| 51 | 193 | 16 | 132 | 138 | 3·2 | 6 |
| 8816 | 154 | 20 | 132 | 132 | 4·0 | 0 |
| 9397 | 127 | 48 | 66 | 177 | 5·9 | 111 |
| 58 | 183 | 12 | 66 | 76 | 2·4 | 10 |
| 2589 | 254 | 10 | 132 | 158 | 5·6 | 26 |

Table 5. *Glucose-utilization rates and glucose spaces measured 4–5 hr. (A), 24 hr. (B) and 48 hr. (C) after feeding*

| Sheep no. | Time interval | Body wt. (kg.) | Average concn. of plasma glucose (mg./100 ml.) | Glucose space (% of body wt.) | Utilization rate (mg./kg./min.) |
|---|---|---|---|---|---|
| 9 | A | 44·0 | 99 | 19 | 2·2 |
| | B | 41·5 | 57 | 30 | 1·8 |
| | C | 40·7 | 45 | 26 | 1·2 |
| 10 | A | 38·3 | 61 | 29 | 2·1 |
| | B | 36·2 | 56 | 25 | 1·6 |
| | C | 38·5 | 54 | 22 | 1·2 |

labelled glucose at a rate of 132 mg./min., glucose made a greater contribution to overall oxidation, values of 33 and 36 % being obtained.

## DISCUSSION

Glucose-utilization rates, which measure the rate of entry into or removal from the glucose pool, are a quantitative estimate of the contribution of glucose to the overall metabolism of the animal. The magnitude of the rates determined in the present investigation, which were equivalent to an entry rate of 90 g./day for a starved sheep of 40 kg. body wt., indicate the central importance of glucose in sheep metabolism.

Only small amounts of glucose are absorbed from the alimentary tract of sheep (cf. Annison & Lewis, 1959) and the origin of the endogenous glucose entering the body pool is of considerable interest (Lindsay, 1959). Propionate is undoubtedly a major precursor, but the available estimates of propionate absorption from the alimentary tract of sheep (Annison, Hill & Lewis, 1957) suggest that the daily absorption rate of propionate is not more than 30–40 g., an amount which would account for only about half of the measured glucose-utilization rate. There is no unequivocal evidence that either acetate or butyrate is glucogenic, and it appears that gluconeogenesis from protein must make a major contribution to the glucose requirements of sheep (Lindsay, 1959). When glucose requirements are assessed, however, it must be noted that utilization rates measured by isotope dilution include entry to the body pool of glucose produced by 'internal' metabolic cycles, e.g.: muscle glycogen → lactate → liver glycogen → glucose → muscle glycogen.

The limitations of single-injection procedures in utilization studies were fully discussed by Steele *et al.* (1956), who concluded that straightforward mathematical treatment of the curve showing the decline in specific activity of plasma glucose after the injection of labelled glucose (cf. Dunn *et al.* 1957) seriously overestimated glucose-utilization rates. The results obtained in the present study with sheep (Table 1) support this view. Skinner, Clark, Baker & Shipley (1959) claimed to have overcome the intrinsic weaknesses of the single-injection method by using a mathematical approach based on the assumption that glucose in the intact animal is distributed throughout a three-compartment system. It seems doubtful, however, whether the parameters governing the entry and outflow of glucose from the multi-compartment system that comprises the intact animal can be defined well enough for adequate mathematical treatment of single-injection data. The difficulties inherent in this approach have been discussed by Sheppard & Householder (1951) and by Hart (1955).

Raised circulating amounts of glucose appeared to inhibit or abolish entry of endogenous glucose, since for four of the five sheep examined calculated utilization rates were roughly equivalent to the rate of infusion of carrier glucose (Table 4). The widely variable apparent glucose spaces measured in these experiments, however, suggested that equilibrium between plasma glucose and the glucose of other body pools had not been fully established. Carrier glucose and [$^{14}$C]glucose, however, were infused as one solution, and during the period of constant plasma glucose concentration and specific activity (Fig. 3) infused glucose and plasma glucose were of almost identical specific activity. Even in the absence of complete mixing of body pools the simplest interpretation of these results is that hepatic entry of glucose was inhibited under glucose load. The rate of infusion of carrier glucose was not large enough to mask changes in endogenous glucose entry, being at most only three times as great as the usual endogenous entry rate. The unchanged specific activity of plasma glucose immediately after the cessation of infusion, which persisted until the plasma glucose concentration fell to the pre-infusion value (Fig. 3), was further evidence of inhibited glucose entry under glucose load, although recycling of labelled glucose and any production of labelled glucose from labelled liver glycogen in this period would tend to nullify the decline in specific activity due to glucose entry. Studies based on the application of glucose load during the decline in blood glucose specific activity in dogs after the injection of [$^{14}$C]glucose (Searle & Chaikoff, 1952; Reichard, Friedmann, Maas & Weinhouse, 1958) suggested that hepatic output of glucose was abolished in response to high blood glucose concentrations, but later it was convincingly shown that the plateau in blood glucose specific activity observed after the application of glucose load was not necessarily due to inhibition of endogenous glucose entry (cf. Steele, 1959). Steele & Marks (1958) produced evidence that hepatic output of glucose in dogs is not inhibited by the sudden application of glucose load. In the present experiments with sheep the procedure allowed time for the animal to adjust to higher circulating concentrations of glucose, but the conclusions must remain tentative until the apparent inhibition of hepatic glucose output can be confirmed by other experimental approaches. A possible explanation of this apparent species difference is that more effective mechanisms for the control of glucose production may exist in sheep, which depend almost entirely on endogenous glucose. The metabolic economy of ruminants appears to be delicately balanced with

respect to glucose availability, particularly in the pregnant or lactating animal (Annison & Lewis, 1959), and any mechanism which conserves glucose, or glucose precursors, is of obvious value.

The relatively small decline in glucose entry in starved sheep (Table 5) indicates the important contribution to gluconeogenesis of sources other than propionate in starvation, since concentrations of volatile fatty acids in the rumen of starved (48 hr.) sheep are low (Annison, 1954) and entry of propionate from endogenous sources is probably negligible. The absence of a correlation between glucose turnover and diet in starved animals maintained on rations differing widely in starch content (Table 1) emphasizes the important role of gluconeogenesis in sheep metabolism.

The continued rise in blood carbon dioxide specific activity during the infusion of labelled glucose was presumably largely due to the conversion of glucose into other metabolites, and their subsequent oxidation, since errors due to incomplete mixing of labelled carbon dioxide with body pool carbon dioxide are minimal after 2–3 hr. (Annison & Lindsay, 1961). This secondary oxidation of glucose implies that estimates of the direct contribution of glucose to oxidative metabolism are almost certainly too high, and should be regarded as maximum values. Moreover, the use of venous blood carbon dioxide in place of expired carbon dioxide when measuring the contribution of labelled substrates to oxidative metabolism may give erroneous results unless mixed venous blood is used, since different tissues may vary considerably in their oxidative capacities. Clearly, jugular blood obtained during the infusion of labelled glucose would be expected to contain carbon dioxide of higher specific activity than that of mixed venous blood, since sheep brain utilizes glucose, but not acetate (McClymont & Setchell, 1956). In the present studies values of 22–23 % and 11–19 % respectively were obtained for the contribution of glucose to total carbon dioxide production in fed and starved animals. These values are much higher than the value (10 %) reported by Baxter *et al.* (1955) in experiments on a lactating dairy cow, but the entry of labelled glucose into milk constituents in this animal makes comparison difficult. In sheep under glucose load about a third of the expired carbon dioxide was derived from pathways involving glucose oxidation. Estimates of the amount of glucose oxidized were not available since total carbon dioxide output was not measured in these experiments.

Steele *et al.* (1956) reported a mean value for the glucose space of fasted dogs of 25 % (range 19–29 %) of body weight, after correction for the glucose content of erythrocytes. In dogs the glucose space was approximately the same as the inulin space (20–25 %), which is a rough measure of the volume of extracellular fluid. The apparent distribution of the glucose pool throughout the extracellular fluid led Steele *et al.* (1956) to suggest that glucose space is a fundamental parameter of glucose metabolism. The values obtained for starved sheep by constant infusion (mean value 23 %, Table 1) were somewhat lower than those reported for dogs, but the relatively larger proportion of body weight represented by the alimentary tract of sheep must be borne in mind. Although a wide range of values for glucose space were obtained in the present experiments (Tables 1 and 3), the results obtained in different experiments on the same animal were in reasonably close agreement. Glucose spaces measured after feeding (Table 3) were significantly higher than those of starved animals (Table 1). Hix, Underbjerg & Hughes (1959) have recently reported values of about 30 % of body weight for the extracellular fluid of sheep, based on thiocyanate distribution, but this estimate included alimentary-tract contents. It would appear therefore that the glucose space of sheep, as measured by the constant-infusion method, is approximately the same as the extracellular space.

When the values for glucose utilization obtained in all constant-infusion experiments in fed and starved sheep were plotted against plasma glucose concentrations a roughly linear relationship was obtained (Fig. 4). This was unexpected in view of the findings of Bing *et al.* (1953) on the relationship between glucose utilization by the human heart and blood glucose concentration. Only small amounts of glucose were extracted at blood glucose concentrations below about 80 mg./100 ml., and glucose utilization reached its maximum value at blood concentrations of 120 mg./ml. The present findings, which were based on the intact sheep, indicate the ability of sheep tissues to utilize glucose at low plasma concentrations, and suggest the absence under these conditions of mechanisms to 'spare' glucose by the preferential oxidation of other substrates. At the highest glucose concentrations obtained there was no evidence of utilization reaching a maximum, but insufficient results were obtained at high plasma glucose concentrations to decide this point.

The wide range of plasma glucose concentrations and glucose-entry rates observed in different animals on similar diets and maintained under similar conditions (Table 1) presumably reflects differences in endocrine balance. These findings emphasize the need for caution when interpreting results obtained on small numbers of animals.

The measurement of the two fundamental parameters of carbohydrate metabolism, glucose-utilization rate and glucose space, constitutes a

valuable tool in the investigation of certain metabolic disorders. This approach is being currently used in this laboratory in the study of ovine pregnancy toxaemia, a condition characterized by impaired carbohydrate metabolism.

## SUMMARY

1. Measurement of glucose utilization in sheep by isotope dilution has indicated the central importance of glucose in sheep metabolism. Glucose-utilization rates in fed and starved (24 hr.) animals, measured by a constant-infusion method, were $2{\cdot}7 \pm 0{\cdot}2(7)$ and $1{\cdot}5 \pm 0{\cdot}09(13)$ mg./min./kg. body wt. respectively. When calculated in terms of metabolic body weight ($W^{0{\cdot}6}$) utilization rates in starved sheep were about 75% of those reported for starved dogs. Corresponding values for the volume of distribution of the body glucose pools, expressed as a percentage of body weight, were $27 \pm 1{\cdot}7(7)$ and $23 \pm 1{\cdot}2(13)$.

2. A comparison of single-injection and constant-infusion methods for the measurement of glucose-utilization rates indicated that significantly higher results were given by the former technique.

3. Evidence of some inhibition of endogenous glucose entry under glucose load was obtained when utilization rates were measured at high concentrations of circulating glucose achieved by constant infusion.

4. A roughly linear relationship was observed when glucose-utilization rates obtained in all constant-infusion experiments were plotted against corresponding plasma glucose concentrations.

5. Rough estimates of the contribution of glucose to overall oxidative metabolism were obtained by a comparison of the specific activities of plasma glucose and blood carbon dioxide. The results suggested that in fed and in starved animals 22–23% and 11–19% respectively of blood carbon dioxide was derived from glucose. Under glucose load this value rose to about 30%.

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# Synthesis of Lipids from [2-$^{14}$C]Acetate and [$^{14}$C]Glucose by *Trichoderma viride*

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The main purpose of this work was to investigate further the synthesis of unsaturated fatty acids, particularly linoleic acid and linolenic acid, by plant tissue. The fungus *Trichoderma viride* was selected as a suitable subject and a study was made of the fate of labelled carbon supplied to it as [2-$^{14}$C]acetate or [$^{14}$C]glucose (generally labelled). In other organisms the synthesis of saturated fatty acids by repeated condensation of $C_2$ units is now well established (Bloch, 1948; Popják, French, Hunter & Martin, 1951), but, although there is evidence for the formation of oleic acid from stearic acid by dehydrogenation in animal tissues (Stetten & Schoenheimer, 1940; Schoenheimer & Rittenberg, 1936; Bernhard, Rothlin & Wagner, 1958), there is much less information about the synthesis of unsaturated fatty acids, particularly in plant tissue. Hilditch (1956) discussed two possible routes of synthesis. The first was a desaturation process starting from stearic acid and proceeding by way of oleic acid and linoleic acid to linolenic acid. The second involved a hydrogenation sequence operating in the reverse direction. Our conclusions, which favour the formation of linoleic acid and linolenic acid by the former process, have been summarized elsewhere (Crombie & Ballance, 1959).

## METHODS

Trichoderma viride *cultures.* Spores were cultivated on agar slopes of a 2% glucose–acetate medium containing (g./l.): $NH_4NO_3$, 5·00; $KH_2PO_4$, 3·50; $MgSO_4,7H_2O$, 2·50; $FeCl_3$, 0·08; $ZnSO_4$, 0·025; agar, 35·00; D(±)-glucose (anhydrous), 20·00; acetic acid, 5·00 (pH adjusted to 6·8 with aq. 50%, w/v, NaOH). The agar suspension was steamed (1 hr.), tubed, autoclaved (10 lb./in.$^2$ for 15 min.), cooled and each tube inoculated with concentrated spore suspension. Incubation at 20° for 10–12 days in continuous light gave rise to an even layer of spores. The spore suspension (10 ml. of sterile water) from each slope was used to inoculate five conical flasks (350 ml.), each containing 50 ml. of the above-mentioned glucose–acetate medium: these experimental mycelium cultures were agitated in the absence of light at 25° on a flask shaker (Ballance, 1961).

*Feeding with radioactive compounds.* The specific activities of the radioactive acetate and glucose stock solutions (0·1 mc/100 ml. of methanol or water respectively) were determined with samples (100 $\mu$l.), which were diluted with 0·5 g. of sodium acetate or 0·25 g. of glucose and then diluted to 10 ml. with water. Samples (100 $\mu$l.) were withdrawn, evaporated on 25 mm. diameter nickel planchets and counted with a gas-flow proportional counter.

After 2 or 4 days' growth on the shaker, experimental mycelium from 20 flasks was suspended in 800 ml. of medium of similar mineral salt concentration but containing, for labelled acetate experiments, 10 g. of glucose/l. and 0·25 g. of sodium acetate/l., and for labelled glucose experiments 5 g. of glucose/l. and 0·125 g. of sodium acetate/l. After equilibration (4 hr.) in the new medium, the radioactive substrate was added. Later the mycelium was harvested, killed in boiling ethanol and the lipid was extracted. The mycelium was aerated with $CO_2$-free air throughout the equilibration and radioactive-feeding periods. In another series of experiments, after the radioactive-feeding period mycelium was grown for a further time in 500–800 ml. of non-radioactive 2% glucose–acetate medium before being killed and analysed.

*Lipid analysis.* Killed mycelium was homogenized in ethanol, the homogenate filtered and the residue washed alternately with ethanol and diethyl ether (final mixture 1:2, v/v). The solvents were evaporated and the total lipid was taken up in light petroleum (b.p. 40–60°).

With ice-cold acetone the lipid was separated into acetone-insoluble (crude phospholipid) and acetone-soluble (crude triglyceride and free fatty acid) fractions. The latter were removed with dilute $Na_2CO_3$ solution and regenerated with HCl. The quality of the acetone fractionation was checked by phosphorus estimations, small samples being saponified, ignited and determined colorimetrically (Hardman & Crombie, 1958). Samples (1–2 mg.) of each lipid fraction were transferred in $CHCl_3$ to nickel planchets and counted (no self-absorption correction was made).

The crude triglycerides and phospholipids were saponified (Crombie & Comber, 1956) and the fatty acids isolated. Reversed-phase column chromatography (Crombie, Comber & Boatman, 1955) was used for both fatty acid-mixture analysis and for the isolation of individual acids. As each acid left the column the solution was made alkaline (NaOH), the acetone was removed by evaporation and traces of liquid paraffin were extracted with diethyl ether. The solution was acidified, the fatty acids were taken up in light petroleum, washed, dried ($Na_2SO_4$) and evaporated, and a sample (1 mg.) was counted for radioactivity. Since oleic acid and palmitic acid were not separated by the reversed-phase column, the figures for these acids were obtained by a second analysis after degradation of the unsaturated acids (including oleic acid) to short-chain-length acids by oxidation with alkaline permanganate (Crombie *et al.* 1955). In addition a sample of each fatty acid mixture was catalyti-

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cally hydrogenated and then chromatographed: the combination of the three chromatographic procedures allows a complete fatty acid analysis to be made.

For samples too small for column chromatography, and for rapid checking of column data, analyses were carried out by the paper-chromatographic technique of Ballance & Crombie (1958): radioautographs were prepared by direct contact with Kodirex X-ray film.

## RESULTS

*Composition of* Trichoderma viride *lipid.* Growth of the fungus under a variety of conditions did not markedly alter the proportion of the lipid fractions (Table 1) or their fatty acid composition. Typical fatty acid analyses are shown in Table 2.

*Gross incorporation of* $^{14}C$ *into the major lipid fractions.* Mycelium (4 days old) from 21 flasks of 2% glucose–acetate culture medium was added to the acetate-feeding medium (750 ml.) and equilibrated for 4 hr. Sodium [2-$^{14}C$]acetate (20 ml. of stock soln., $12{\cdot}7 \times 10^6$ counts/min.) in sterile water was added, and, after 1 hr., the mycelium was filtered off and analysed. Identical runs, which were set off at the same time, were harvested after 3 and 5 hr. The incorporation of $^{14}C$ into the crude phospholipid, crude triglyceride and free fatty acid fractions (Fig. 1) gave no indication that one of the three fractions served as a precursor for either of the others.

*Incorporation of* $^{14}C$ *into the individual fatty acids of the major lipid fractions.* The individual fatty acids from the phospholipid and triglyceride fractions of the above-described experiments were isolated by column chromatography and their specific activities were determined (Table 2). In both fractions the $^{14}C$ rapidly accumulated in palmitic acid, oleic acid and stearic acid, particularly in palmitic acid. Accumulation in linoleic acid is considerably slower than in oleic acid, but faster than in linolenic acid. The three corresponding samples of free fatty acids were too small for column-chromatographic analysis, but on radioautographs most of the blackening appeared at the combined oleic acid–palmitic acid spot. In further

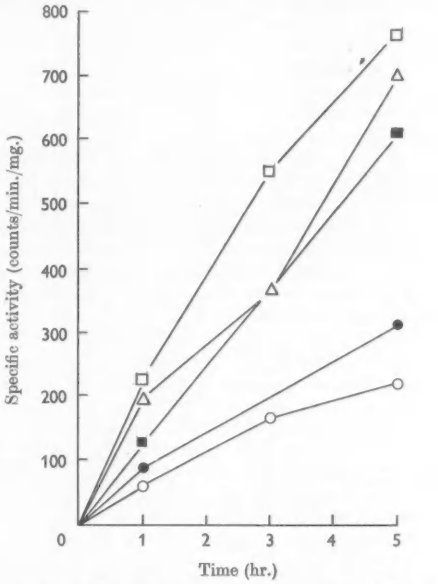


Fig. 1. Incorporation of radioactive acetate into the major components of *Trichoderma* lipid. □, Phospholipid; △, triglyceride; ■, free fatty acids; ●, acetone-soluble non-saponifiable material; ○, acetone-insoluble non-saponifiable material.

Table 1. *Major lipid components of* Trichoderma viride *grown under different conditions*

| Experimental conditions | Age (days) | Dry wt. (g./flask or tube) | Lipid (g./100 g. dry wt.) | Acetone-insoluble phospholipid* (g./100 g. of lipid) | Acetone-soluble triglyceride† (g./100 g. of lipid) | Free fatty acids (g./100 g. of lipid) | Non-saponifiable (g./100 g. of lipid) |
|---|---|---|---|---|---|---|---|
| Spores (20% glucose–agar slopes) | 12 | 0·017 | 9·6 | 21 (82) | 64·3 (4·0) | 14·5 | 6·2 |
| Mycelium (aerated 20% glucose soln.) | 7 | 0·43 | 4·8 | 61·6 (79) | 37·4 (6·0) | 1·0 | 14·0 |
| Mycelium (non-aerated 20% glucose soln.) | 14 | 0·22 | 5·4 | 54·9 (99) | 35·5 (1·0) | 9·6 | 12·0 |
| Mycelium (7 days in aerated 20% glucose soln., then 7 days in aerated water) | 14 | 0·48 | 5·3 | 40·7 (88) | 54·3 (5) | 5·0 | 6·3 |
| Mycelium (aerated 2% glucose–acetate soln.) | 4 | 0·44 | 7·6 | 45·7 (79) | 52·7 (8) | 1·6 | 0·6 |

* Percentage purity (given in parentheses) was obtained from 3·8 μg. of P≡100 μg. of phospholipid.
† Percentage phospholipid contaminant was obtained from phosphorus determinations as described above.

Table 2. *Distribution of* $^{14}C$ *among fatty acids synthesized from* [2-$^{14}C$]*acetate*

| Fatty acid* (mg./100 mg.) | | Specific activities† (counts/min./mg.) 1 hr. | 3 hr. | 5 hr. |
|---|---|---|---|---|
| Phospholipid fraction | | | | |
| Linolenic | 2·8 | 25 | 83 | 116 |
| Linoleic | 53·9 | 36 | 153 | 254 |
| Oleic | 20·4 | 219 | 971 | 1519 |
| Palmitic | 19·8 | 485 | 1409 | 2463 |
| Stearic | 1·8 | 74 | 743 | 1978 |
| Behenic | 1·4 | 89 | 100 | 422 |
| Triglyceride fraction | | | | |
| Linolenic | 4·6 | 27 | 67 | 21 |
| Linoleic | 38·6 | 28 | 99 | 197 |
| Oleic | 32·7 | — | 272 | 631 |
| Palmitic | 13·9 | 114 | 912 | 561 |
| Stearic | 8·7 | 119 | 252 | 292 |
| Behenic | 1·5 | 102 | 20 | 29 |

* Composition of the fatty acid mixtures remained steady throughout the 5 hr. period.
† Fatty acids obtained from lipid fractions shown in Fig. 1.

Table 3. *Effect of varying substrate and age of culture on the distribution of* $^{14}C$ *among the total synthesized fatty acids*

| Fatty acids | [$^{14}C$]Glucose specific activity (counts/min./mg.) | 2-day Culture: fatty acid composition (mg./100 mg.) | [2-$^{14}C$]Acetate: specific activity (counts/min./mg.) | 2-day Culture: fatty acid composition (mg./100 mg.) | [2-$^{14}C$]Acetate: specific activity (counts/min./mg.) | 4-day Culture: fatty acid composition (mg./100 mg.) |
|---|---|---|---|---|---|---|
| Linolenic | 65 | 2·2 | 434 | 2·2 | 48 | 5·0 |
| Linoleic | 53 | 42·3 | 1522 | 42·3 | 149 | 44·0 |
| Oleic | 329 | 29·0 | 5806 | 29·0 | 701 | 24·0 |
| Palmitic | 508 | 20·9 | 9628 | 20·9 | 1129 | 21·3 |
| Stearic | 367 | 5·4 | 2570 | 5·4 | 940 | 5·6 |
| Total activity supplied (counts/min.) | $54·3 \times 10^6$ | | $17·4 \times 10^6$ | | $14·5 \times 10^6$ | |

work the separation into phospholipid, triglyceride and free fatty acids was not carried out, but the component fatty acids were examined as a whole. Table 3 compares the use of [$^{14}C$]glucose and [2-$^{14}C$]-acetate as substrates for a 2-day-old culture and the effect of [2-$^{14}C$]acetate feeding to a 4-day-old culture. The pattern of incorporation is similar to that in Table 2. Tables 2 and 3 emphasize that the extent of labelling does not correspond with quantity of the acid that occurs. For instance, linoleic acid, which represents 40–50 % of each fatty acid mixture, accumulates only 8–20 % of the radioactivity incorporated into the whole mixture.

A small acid peak that emerged from the reversed-phase columns ahead of linolenic acid (the first to emerge) had low specific activity. It is thought to represent the oxidation product of one or more of the unsaturated acids [cf. Schlenk, Gellerman, Tillotson & Mangold (1957), who noted radioautographic peaks which accumulated at the solvent front during study of the auto-oxidative breakdown of radioactive methyl esters of oleic acid, linoleic acid and linolenic acid].

Table 4. *Specific activity (counts/min./mg.) of fatty acids after growth of mycelium (for* 3 *hr.) in radioactive acetate medium and continued growth (to* 20 *hr.) in non-radioactive medium (*4*-day-old mycelium)*

| Fatty acids | Times of sampling (hr.) 3* | 7 | 15 | 20* |
|---|---|---|---|---|
| Total fatty acids | 630 | 689 | 555 | 518 |
| Linolenic | 48 | 33 | 76 | 95 |
| Linoleic | 149 | 146 | 277 | 213 |
| Oleic + palmitic | 902 | 704 | 716 | 585 |

* Percentage composition (g./100 g. of fatty acid) at 3 hr. (and at 20 hr., in parentheses): oleic acid + palmitic acid, 47·1 (43·2); linoleic acid, 40·5 (42·5); linolenic acid, 4·5 (4·4).

*Consequences of transferring mycelium from* $^{14}C$*-labelled to unlabelled substrate.* Mycelium (4 days old; 270 g. fresh wt.) was equilibrated (4 hr.) with acetate-feeding medium (2·4 l.); sodium [2-$^{14}C$]-acetate ($43·4 \times 10^6$ counts/min.) was added and metabolism allowed to continue for 3 hr. The mycelium was filtered, thoroughly washed and transferred to non-radioactive 2 % glucose–acetate medium. Mycelium was harvested in 45 g. portions

3, 7, 15 and 20 hr. from the introduction of the labelled acetate. The total fatty acids were isolated from each portion and, as is shown in Table 4, there was first a rise and then a slight decline in activity consequent on transfer to the non-radioactive medium. Examination of the specific activities of the individual fatty acids in Table 4 reveals again this rise and slight fall in activity. Thus first oleic acid and palmitic acid show a decline in activity, linoleic acid shows a rise and decline and linolenic acid still shows a rise in activity after 20 hr. It may be inferred at this stage that oleic acid and palmitic acid (formed before the others) might be undergoing conversion first into linoleic acid then into linolenic acid. It is difficult to explain the fact that at 7 hr. the specific activity of the total fatty acids had risen, whereas all the values of the individual acids (except stearic acid and behenic acid, whose specific activities were not determined in this experiment) had fallen, compared with their 3 hr. values; however, these changes in the labelling of the acids (Table 4) are not great and are therefore more subject to experimental error than are the changes (in the labelling pattern) which were much more forcibly demonstrated with the more actively metabolizing 2-day-old mycelium.

Twenty-four 2-day-old mycelial mats were equilibrated (4 hr.) in aerated acetate-feeding medium (600 ml.). Sodium [2-$^{14}$C]acetate ($17{\cdot}36 \times 10^6$ counts/min.) was added and growth allowed to proceed for 3 hr. The mycelium was harvested; the total fatty acids were extracted and analysed. A second sample of 24 mycelial mats was treated in exactly the same manner, but, instead of being harvested, was thoroughly washed and growth was continued in non-radioactive 2% glucose–acetate medium for 2 days more, before harvesting and analysis occurred. The results from both these experiments have been compared in Table 5.

Total radioactivity in the fatty acids at the end of the [2-$^{14}$C]acetate feeding (Table 5) was $43 \times 10^4$ counts/min., but, after continued growth in non-radioactive medium, increased to $71 \times 10^4$ counts/min. When 4-day-old mycelium was transferred from active to non-active culture conditions, a slight rise in activity and then a decline took place; here the increase in activity is marked and no decline has occurred. The more marked increase in activity is thought to result from the high speed of metabolism of the 2-day-old mycelium, coupled with the presence of a large fatty acid-precursor pool within the organism. Simmons & Quackenbush (1954) encountered a similar effect in work on soya bean, and offered the same explanation.

The increase in activity, however, does not obscure the substantial movement of the $^{14}$C label into linoleic acid and the decline in label present in palmitic acid. During this change in the labelling pattern the percentage of linoleic acid by weight has remained stationary, but palmitic acid has declined and oleic acid has risen. These results were confirmed by paper chromatography and the preparation of radioautographs. During the period between 2 and 4 days of growth, considerable fatty acid synthesis took place; the average weight of the total fatty acids from 24 mycelial mats was 253 mg. at 2 days, and had increased to 418 mg. at the end of 4 days.

The position with respect to feeding with generally labelled [$^{14}$C]glucose is similar to that found for [2-$^{14}$C]acetate. Labelled glucose ($54 \times 10^6$ counts/min.) was introduced to 2-day-old mycelium (from 24 flasks) contained in glucose-feeding medium (400 ml.) and growth allowed to continue for 3 hr. Three-quarters of the mycelium was killed and analysed. The remainder was returned after washing to non-radioactive 2% glucose–acetate-feeding medium for a further 2 days and then harvested and analysed. After growth for 3 hr. on radioactive glucose 10 times as much of the incorporated radioactivity had appeared in the oleic acid and palmitic acid as had entered linoleic acid. On further growth for 2 days, the incorporation of $^{14}$C into linoleic acid is greatly increased, whereas that of the oleic acid and palmitic acid fraction altered only a little

Table 5. *Distribution of* $^{14}$C *in fatty acids from 2-day-old mycelium grown (3 hr.) in a radioactive acetate medium and then allowed to grow for 2 days in non-radioactive medium*

| | Before continued growth | | | After continued growth | | |
|---|---|---|---|---|---|---|
| Fatty acid | Composition (mg./100 mg.) | $10^2 \times$ Specific activity (counts/min./mg.) | $10^2 \times$ Total activity (counts/min.) | Composition (mg./100 mg.) | $10^2 \times$ Specific activity (counts/min./mg.) | $10^2 \times$ Total activity (counts/min.) |
| Linolenic | 2·2 | 4·3 | 9·5 | 0·7 | 23·1 | 17·1 |
| Linoleic | 40·4 | 15·2 | 614·7 | 39·9 | 59·4 | 2370·9 |
| Oleic | 27·7 | 58·1 | 1608·3 | 38·2 | 84·4 | 3224·5 |
| Palmitic | 20·0 | 96·3 | 1921·8 | 13·9 | 96·1 | 1336·1 |
| Stearic | 5·2 | 25·7 | 133·4 | 5·5 | 29·2 | 160·0 |
| Arachidic* | 1·0 | 8·9 | 9·3 | 0·7 | 9·7 | 6·4 |
| Behenic | 3·5 | 4·0 | 14·1 | 1·2 | 4·9 | 5·9 |

* Formation of arachidic acid seems to be associated with high acetate concentration and conditions of good aeration.

Table 6. *Distribution of* $^{14}C$ *in fatty acids from 2-day-old mycelium grown (3 hr.) in the presence of* [$^{14}C$]*glucose and transferred for a further 2 days to a non-radioactive medium*

| | Before transfer | | After transfer | |
|---|---|---|---|---|
| Fatty acids | Composition (mg./100 mg.) | $10^2 \times$ Total activity (counts/min.) | Composition (mg./100 mg.) | $10^2 \times$ Total activity (counts/min.) |
| Linolenic | 2·1 | 1·4 | 0·7 | 1·0 |
| Linoleic | 40·4 | 21·4 | 40·4 | 160·4 |
| Oleic + palmitic | 47·3 | 192·3 | 52·0 | 174·7 |

(Table 6). The composition (mg./100 mg.) of the fatty acid mixture did not change significantly during the experiment.

## DISCUSSION

In considering the synthesis of the major fractions of *Trichoderma* lipid, it seems from the specific activities of the fractions (Fig. 1) that deacylation of the fatty acyl-coenzyme A to form free fatty acids is not the major pathway to phospholipids and triglycerides; the latter fractions probably arise directly from activated fatty acids.

The finding that *Trichoderma* incorporated radioactive carbon chiefly into palmitic acid is consistent with results obtained by Wakil (1958), Wakil & Ganguly (1959), Long & Porter (1959), Martin, Horning & Vagelos (1960) and Kusunose, Kusunose, Kowa & Yamamura (1960). The enzyme systems used by these groups of investigators all gave rise initially to highly labelled palmitic acid. It would appear (Table 7) that in *Trichoderma* the remaining saturated fatty acids arise by addition of one or more activated coenzyme A acetate or $C_2$ units to the first-formed palmitic acid.

Throughout the experiments with *Trichoderma*, palmitic acid always had an initially higher specific activity than oleic acid. On transferring mycelium from labelled to unlabelled substrate, the specific activity of oleic acid rose relative to the level of activity in palmitic acid, a result which could suggest a conversion of palmitic acid into oleic acid. This conversion would be expected to be by way of stearic acid, the desaturation of which has been demonstrated in animal tissue by Schoenheimer & Rittenberg (1936), Stetten & Schoenheimer (1940) and by Bernhard *et al.* (1958). More recently Bu'Lock & Ridyard (1960) described the conversion of [9:10-$^{14}C_2$]stearic acid into [9:10-$^{14}C_2$]oleic acid in rabbit mammary gland tissue, and, although this conversion did not occur in germinating peanut cotyledons, Bloomfield & Bloch (1960) showed the desaturation of stearic acid to oleic acid in cell-free yeast extracts. In the present work, however, the low specific activity of stearic acid implies that this acid, as such, is not an intermediate in the rapid conversion of palmitic acid into oleic acid. A similar finding was made by Dittmer & Hanahan (1959), but this does not exclude the possibility of activated or modified stearic acid being an intermediate between activated palmitic acid and oleic acid. Later work by the same authors (Hanahan & Dittmer, 1960) revealed that when labelled palmitic acid was fed to rats, a very rapid conversion into stearic acid and thence into oleic acid took place; unfortunately no experimental details were given.

Table 7. *Specific activities (counts/min./mg.) of the saturated fatty acids from 2-day-old mycelium grown for 3 hr. in the presence of labelled substrate*

| | No. of carbon atoms | Radioactive substrate | |
|---|---|---|---|
| Fatty acid | | Glucose | Acetate |
| Palmitic | 16 | 508 | 9628 |
| Stearic | 18 | 367 | 2570 |
| Arachidic | 20 | 121 | 890 |
| Behenic | 22 | 81 | 460 |

The use of triose units derived from glucose to synthesize linolenic acid, and the subsequent successive hydrogenation of linolenic acid to linoleic acid to oleic acid has been discussed by Hilditch (1956). But when *Trichoderma* was grown on labelled glucose there was only a slight incorporation of radioactivity; what little did enter the fatty acids was found initially mainly in palmitic acid and oleic acid, suggesting that the glucose had given rise to acetate units which resulted in the typical palmitic acid/oleic acid-labelling pattern normally produced by feeding with labelled acetate.

The arguments in favour of the introduction of one or two double bonds into the oleic acid molecule are based on the change in the labelling pattern that took place when *Trichoderma* mycelium containing strongly labelled oleic acid and palmitic acid was transferred (for 2 days) to unlabelled substrate. Only when such a transfer was effected did the specific activity of linoleic acid reach a value similar to that of palmitic acid or oleic acid. During the 2 days of continued growth on unlabelled substrate 40 % of the fatty acids synthesized was linoleic acid, and it is therefore reasonable to postulate a conversion of palmitic acid or oleic (most probably oleic) acid into linoleic acid. The increased specific activity of linolenic acid could be interpreted as a further

dehydrogenation of linoleic acid. The alternate labelling of carbon atoms of both saturated and unsaturated long-chain fatty acids discovered by Gibble & Kurtz (1956) when flax was fed with [$^{14}$C]-acetate can be construed as evidence favouring dehydrogenation of saturated fatty acids. The only apparent alternative to the dehydrogenation pathway would be a route direct from acetate to linoleic acid, the reactions for which were slow to restart after the transfer of mycelium from the original medium to the feeding medium. This route appears highly unlikely on several counts. In our view the continued synthesis of linoleic acid from unlabelled oleic (or less likely palmitic) acid is the best explanation of the initial low specific activity of linoleic acid.

Since completion of the above-described work and publication of our preliminary communication (Crombie & Ballance, 1959) these deductions have received support from Yuan & Bloch (1960), who have described the production of labelled linoleic acid from labelled oleic acid in *Torulopsis utilis*.

## SUMMARY

1. Phospholipids closely followed by triglycerides had higher specific activities than the free fatty acids in the lipid obtained from 4-day-old *Trichoderma* mycelium that had been grown for 1, 3 or 5 hr. in the presence of [2-$^{14}$C]acetate.

2. After only 1 hr. in the presence of labelled acetate, radioactive carbon was found in all the long-chain fatty acids, especially oleic acid and palmitic acid, in all three major fractions of the lipid.

3. When the 4-day-old mycelium containing high-specific-activity palmitic acid and oleic acid was transferred to unlabelled substrate there was slight indication of conversion of these acids into linoleic acid and linolenic acid.

4. When 2-day-old mycelium, containing high-specific-activity palmitic acid and oleic acid (this activity was obtained from either [$^{14}$C]acetate or [$^{14}$C]glucose), was transferred to an unlabelled medium for 2 days the specific activity of linoleic acid (representing 40–50 % of the fatty acids present), which was always initially very low, rose to a value equal to the activities of palmitic acid and oleic acid.

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# The Lipid Composition of Rat Liver

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In a previous publication (Getz & Bartley, 1961) the fatty acid compositions of various intracellular compartments of the rat liver were investigated. This work has now been extended to the fatty acid composition of the different lipid classes of the liver. The data obtained will be used as a basis for comparison of the lipid composition of each of the cell components. This paper reports a compositional analysis of the whole rat liver.

## EXPERIMENTAL

*Fractionation of lipids on silicic acid.* The techniques of preparation of the rat livers, of extraction of the lipid by chloroform–methanol (2:1, v/v) and of determination of the fatty acid composition were as described by Getz & Bartley (1961). The lipid extract was chromatographed on a silicic acid column (20 g. of silicic acid) prepared according to the method of Hirsch & Ahrens (1958), except that the Mallinckrodt silicic acid (mesh 100) used was not pre-ground before size-grading. The lipid load on the column was never more than 250 mg. of lipid, i.e. 12·5 mg./g. of adsorbent. Lipids were loaded on in light petroleum (b.p. 60–80°) and were eluted stepwise with the solvents shown in Table 1. The elution data presented by Hirsch & Ahrens (1958) for neutral lipids and by Hanahan, Dittmer & Warashina (1957) for phospholipids were used as the guide for the choice of these solvent-elution sequences and volumes. However, it was necessary to determine the volumes of chloroform–methanol (3:2) required to separate the inositol from the lecithin. This was done by collecting fractions and measuring their phosphorus content. The separation is shown in Fig. 1. Hence the first 100 ml. of the chloroform–methanol (3:2) was collected separately from the remaining 400 ml.

The composition of the silicic acid fractions was checked by paper chromatography on silicic acid-impregnated paper according to Marinetti, Erbland & Kochen (1957). The results showed that the stepwise elution used produced the same mixtures of lipids as were found by Hanahan *et al.* (1957). Each of the solvent fractions was evaporated to dryness under $N_2$ and made up to 10 or 25 ml. with chloroform before storage at −20° until required.

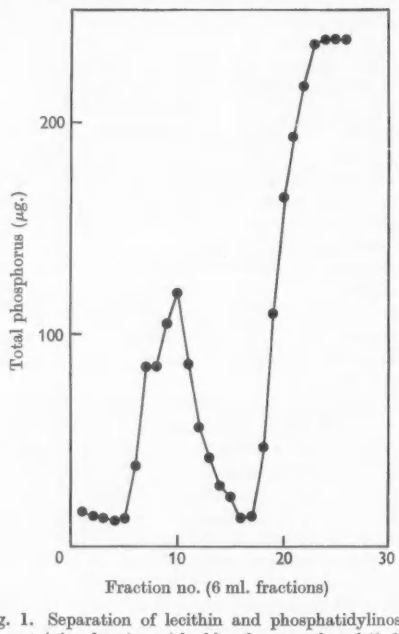


Fig. 1. Separation of lecithin and phosphatidylinositol-containing fraction with chloroform–methanol (3:2).

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Table 1. *Fractionation of lipids on silicic acid*

| Fraction no. | Solvent mixture | Main fraction expected |
|---|---|---|
| 1 | 350 ml. of 1% ether in light petroleum (b.p. 60–80°) + 60 ml. of 4% ether in light petroleum | Cholesterol ester |
| 2 | 400 ml. of diethyl ether | Triglyceride |
| 3 | 225 ml. of chloroform–methanol (7:1, v/v) | Serine phosphatide |
| 4 | 200 ml. of chloroform–methanol (4:1, v/v) | Ethanolamine phosphatide + serine phosphatides |
| 5 | 100 ml. of chloroform–methanol (3:2, v/v) | Inositol and serine phosphatide |
| 6 | 400 ml. of chloroform–methanol (3:2, v/v) | Lecithin |
| 7 | 200 ml. of chloroform–methanol (1:4, v/v) | Sphingomyelin |

Table 2. *Lipids from rat liver*

22·9% of the rat-liver pulp consisted of solids precipitable with trichloroacetic acid. All results are given as μmoles/g. dry wt. of homogenate.

| Fraction no. | Eluting solvent | Lipid esters in fraction (μmoles) | Total phosphate in fraction (μmoles) | Ester/phosphate | Total choline in fraction (μmoles) | Choline/phosphate | Total amino N in fraction (μmoles) | Amino N/phosphate |
|---|---|---|---|---|---|---|---|---|
| 1 | 1% of diethyl ether in light petroleum (60–80°) followed by 4% of ether in light petroleum | 15·1 | — | — | — | — | — | — |
| 2 | 100% ether | 67·6 | — | — | — | — | — | — |
| 3 | Chloroform–methanol (7:1) | 14·7 | 18·0 | 0·8 | — | — | 14·0 | 0·8 |
| 4 | Chloroform–methanol (4:1) | 55·8 | 28·6 | 2·0 | 0·23 | 0·008 | 27·8 | 1·0 |
| 5 | Chloroform–methanol (3:2) | 26·6 | 15·7 | 1·7 | 0·48 | 0·03 | 3·6 | 0·2 |
| 6 | Chloroform–methanol (3:2) | 159·0 | 88·5 | 1·8 | 64·0 | 0·7 | 2·0 | 0·02 |
| 7 | Chloroform–methanol (1:4) | 2·4 | 4·15 | 0·5 | 0·47 | 0·1 | 0·5 | 0·1 |
| Total in all fractions | | 341 | 155 | — | 65·18 | — | 48·0 | — |
| Total from sample before fractionation | | 338 | 139·5 | — | — | — | — | — |
| Recovery (%) | | 100·05 | 111 | — | — | — | — | — |

Barron & Hanahan (1958) described an improved procedure for the fractionation of neutral lipids. This was used for a further batch of rat livers. The lipid extract was made with ethanol and ether as described by Getz & Bartley (1961). The lipid extract was purified according to Folch, Lees & Sloane-Stanley (1957). Unmodified Mallinckrodt silicic acid (mesh 100) was used for the separation and the column was prepared, packed and washed according to Barron & Hanahan (1958). For elution, 15% of benzene in hexane was used for the sterol esters, 15% of diethyl ether in hexane for the triglycerides and diethyl ether alone for the remaining neutral glycerides. After the elution of the neutral lipid fractions, the phospholipids were eluted with chloroform–methanol mixtures in the sequence indicated above, with one exception. In this, chloroform–methanol (9:1) (100 ml.) replaced the chloroform–methanol (7:1). The fraction eluted by this solvent is referred to in Tables 3 and 5 as 'non-polar phospholipid'. The chromatographic separation was followed by a semi-quantitative visual assessment of the esters on a small portion of each 10 ml. fraction collected on the fraction collector. The ester determination was that of Rapport & Alonzo (1955) except that only 0·5 ml. of the ferric perchlorate was added to each tube to increase the sensitivity of the determination. Methyl esters of fatty acids were prepared by the inter-esterification and micro-sublimation technique of Stoffel, Chu & Ahrens (1959).

*Solvents.* All solvents were AnalaR grade, except for ethanol and methanol, which were used without further purification, and hexane, which was the 67–69° boiling fraction distilled from crude laboratory hexane.

*Analytical methods.* Measured portions of the fractions were taken for the determination of: (*a*) Lipid esters (method of Rapport & Alonzo, 1955). (*b*) Total phosphorus (method of Berenblum & Chain, 1938, as modified by Bartley, 1953). (*c*) Amino N (method of Cocking & Yemm, 1954). Serine was used as standard and the result was multiplied by 10/9 to account for the lower colour yield for intact phosphatidylserine shown by Lea & Rhodes (1955). (*d*) Choline (method of Wheeldon & Collins, 1958, modified slightly in order to shorten the time required for preparing the hydrolysate). After hydrolysis, the hydrolysate was quantitatively filtered and the filtrate dried *in vacuo* to remove excess of HCl. The residue was dissolved in less than 1 ml. of water and the solution was transferred to a graduated tube. After neutralization with N-NaOH to pH 6–7, the solution was extracted twice with diethyl ether to remove fatty acid and the volume was made to 1 ml. A sample (0·5 ml.) of this solution was taken for the choline estimation but 0·5 ml. of phosphomolybdate was used in place of the 0·1 ml. suggested by Wheeldon & Collins (1958). (*e*) Cholesterol (method of Zlatkis, Zak & Boyle, 1953, as modified by Henry, 1957). (*f*) Glycerol (method of Hanahan & Olley, 1958, 2N-HCl being used for hydrolysis). More usually the glycerol was estimated in the water-soluble residue remaining after the light-petroleum extraction of the inter-esterification reaction mixture but without removing the HCl.

*Gas–liquid chromatography.* The techniques were those described by Getz & Bartley (1961).

## RESULTS

For the fractionation of all the lipid classes of the liver 10·8 g. of liver pulp was extracted. This yielded 2·47 g. of material precipitable by trichloroacetic acid. The total extractable lipids constituted 18·55% of this dry weight. For the fractionation of the neutral lipids 9·44 g. of liver pulp was extracted. The dry weight was 2·11 g. and the lipid was 23·4% of this dry weight. The results of the silicic acid fractionations are given in Tables 2 and 3, and of the fatty acid determinations in Tables 4 and 5. Table 2 shows that phosphatidylcholine is the major lipid of the rat liver. It is assumed in silicic acid fraction 5 that the difference between the total phosphate and the sum of amino nitrogen and choline in this fraction is due to phosphoinositides. Some 70% of the total phosphate in this fraction when calculated on this basis is accounted for as phosphoinositides. The conclusion is supported by the fact that stearate and arachidonate predominate amongst the fatty

Table 3. *Neutral lipid from livers of young rats*

22·3% of the rat-liver pulp consisted of solids precipitable with trichloroacetic acid. All results are given as μmoles/g. dry wt. of homogenate.

| Fraction no. | Eluting solvent | Lipid esters in fraction (μmoles) | Total phosphate in fraction (μmoles) | Total amino N in fraction (μmole) | Total glycerol in fraction (μmoles) | Ester/glycerol | Ester/phosphate | Amino N/phosphate |
|---|---|---|---|---|---|---|---|---|
| 1 | 15% of benzene in hexane | 5·12 | — | 0·13 | 0·19 | 23·7 | — | — |
| 2 | 15% of hexane in ether | 35·6 | 0·13 | 0·39 | 17·7 | 2·01 | — | — |
| 3 | 100% ether | 7·8 | 0·18 | 0·57 | 3·24 | 2·42 | — | — |
| 4 | Chloroform–methanol (9:1) (non-polar phospholipid) | 20·0 | 3·96 | 0·66 | — | — | 5·04 | 0·17 |

Table 4. *Fatty acid composition of lipids from rat liver*

'Present' indicates that the fatty acid is present as less than 0·02% of the total acids. Analysis of the non-fatty acid portion of the lipids is given in Table 2. For further details see text. Results are given as g./100 g. of fatty acid methyl ester. Fatty acid designation: br., branched; hbr., highly branched; uns., unsaturated acid (no. of double bonds unknown); a, acids identified by comparison with authentic standards; b, acids identified by comparison of their retention volumes with those in the literature. Remaining acids are designated solely on the basis of retention volumes on two stationary phases.

| | Main lipid class in material analysed | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Acid | Total esters | Sterol esters | Triglycerides | Serine phosphatide | Serine + ethanolamine phosphatides | Serine phosphatides + inositides | Lecithin | Sphingomyelin | Extracted free acid |
| 9-br. | 0·05 | — | — | — | — | — | — | — | — |
| 11-br. | — | Present | Present | — | — | 0·02 | — | — | — |
| 12-br. | — | — | 0·04 | — | — | — | — | Present | — |
| a 12-0 | 0·06 | Present | 0·15 | Present | Present | Present | — | 0·51 | 0·31 |
| 12-1 | — | — | Present | — | — | — | — | — | — |
| 13-br. | — | 0·02 | — | — | — | 0·06 | — | Present | — |
| b 13-0 | 0·08 | — | Present | — | Present | — | — | — | — |
| a 14-0 | 0·82 | 0·70 | 1·05 | 0·86 | 0·08 | 0·05 | 0·46 | 0·71 | 0·40 |
| 14-br. | — | — | — | — | Present | Present | — | Present | 0·61 |
| 14-br. | — | — | — | — | Present | — | — | — | — |
| b 14-1 | 0·04 | 0·06 | Present | 0·20 | 0·04 | — | — | 0·99 | — |
| b 15-0 | 0·50 | 0·58 | 0·57 | 0·61 | 0·17 | — | 0·34 | 0·93 | 0·78 |
| 15-br. | — | 0·08 | Present | — | Present | 0·06 | — | 0·13 | — |
| 15-br. | 0·21 | 0·64 | — | 0·33 | — | Present | — | — | — |
| a 16-0 | 25·00 | 26·05 | 25·85 | 22·80 | 25·70 | 12·63 | 29·70 | 20·40 | 30·60 |
| b 16-1 | 1·90 | 1·92 | 2·92 | 2·24 | 0·47 | 0·30 | 0·96 | 0·99 | 6·77* |
| 16-br. | — | 0·42 | Present | 0·15 | Present | — | — | Present | 0·44 |
| b 17-0 | 0·25 | 0·47 | Present | 0·83 | 0·72 | 0·70 | 0·63 | Present | 0·89 |
| 17-br. | — | 0·52 | Present | 0·36 | 0·08 | 0·15 | 0·38 | — | — |
| 18-hbr. | — | 0·35 | Present | — | 0·07 | — | 0·15 | — | — |
| a 18-0 | 11·90 | 2·68 | 1·47 | 15·25 | 20·80 | 38·60 | 16·80 | 11·40 | 10·45 |
| a 18-1 | 20·50 | 40·70 | 28·50 | 15·00 | 8·11 | 5·47 | 10·42 | 11·40 | 25·00 |
| a 18-2 | 22·80 | 17·70 | 29·60 | 30·85 | 13·40 | 5·78 | 16·75 | 49·60* | 19·45 |
| a 18-3 | — | — | 0·86 | — | — | — | — | — | — |
| 19-br. | — | — | — | — | 0·13 | 0·63 | — | — | — |
| b 20-1 | 0·30 | 0·31 | — | — | Present | — | — | — | — |
| b 20-2 | — | — | Present | Present | Present | 0·74 | — | Present | — |
| b 20-3 | 0·20 | 0·49 | 0·30 | 0·77 | 1·07 | 1·86 | 1·54 | Present | — |
| b 20-4 | 9·80 | 1·86 | 1·10 | 7·60 | 19·85 | 28·20 | 18·30 | 3·04 | 4·35 |
| b 20-5 | 0·54 | — | 1·34 | — | — | — | — | — | — |
| b 22-5 | 0·50 | 0·30 | 2·78 | — | 0·90 | 0·91 | Present | — | — |
| b 22-6 | 3·71 | 3·42 | 2·93 | 2·00 | 8·50 | 3·86 | 3·43 | — | — |
| 24-uns. | 0·67 | — | 0·69 | — | — | — | — | — | — |

* Peak contains more than one compound.

Table 5. *Fatty acid composition of neutral lipids from young rat liver*

Details are as given in Table 3 and text. Results are given as g./100 g. of fatty acid methyl ester. Fatty acid designation (a and b) is as given in Table 4.

| | Main lipid class in material analysed | | | | |
|---|---|---|---|---|---|
| Acids | Total esters | Sterol ester | Triglycerides | Diglycerides | Non-polar phospholipid |
| $C_{10}$–$C_{12}$ | — | — | — | — | 0·60 |
| a 14-0 | 0·23 | 0·58 | 0·52 | — | 0·41 |
| b 14-1 | — | Present | — | — | — |
| b 15-0 | 0·36 | 0·57 | 0·43 | 0·90 | 0·28 |
| 16-br. | 0·05 | 1·46 | 0·27 | — | 0·18 |
| a 16-0 | 22·60 | 21·20 | 25·00 | 23·90 | 8·94 |
| b 16-1 | 1·47 | 5·47 | 2·66 | 1·61 | 2·84 |
| b 17-0 | 0·53 | 1·02 | 0·30 | — | 0·32 |
| b 17-br. | 0·20 | — | 0·36 | — | — |
| 17-uns. | — | 1·78 | 0·22 | — | — |
| 18-hbr. | 0·07 | — | — | — | — |
| a 18-0 | 15·10 | 12·25 | 2·33 | 6·56 | 3·64 |
| a 18-1 | 17·65 | 27·05 | 29·40 | 28·80 | 15·60 |
| a 18-2 | 20·00 | 17·96 | 29·40 | 34·10 | 62·00 |
| a 18-3 | — | 0·25 | 0·66 | — | 0·49 |
| b 19-0 | — | — | 0·29 | — | — |
| 19-br. | 0·25 | — | — | — | — |
| b 20-1 | — | 0·44 | 0·61 | — | — |
| b 20-2 | 0·53 | 0·11 | 0·30 | — | 0·74 |
| b 20-3 | 0·95 | 0·03 | — | — | 1·10 |
| b 20-4 | 15·11 | 7·55 | 1·68 | 4·20 | 3·12 |
| b 20-5 | — | 0·66 | 1·11 | — | — |
| 22-5 | — | 0·98 | 1·74 | — | — |
| b 22-5 | 1·03 | 0·58 | 2·71 | — | — |
| b 22-6 | 4·04 | — | — | — | — |

acids in this fraction. These two fatty acids have already been reported as major constituents of phosphoinositides (Dittmer & Hanahan, 1959). The low choline : phosphorus ratio in the lecithin fraction has no obvious explanation.

The fatty acid-ester pattern of each lipid class showed characteristic differences. One point of similarity was the rather similar percentage of palmitate in each lipid class with the exception of the inositide, which had only about half the palmitate concentration (12 % compared with about 25 %). The neutral-lipid fractions were characterized by high palmitoleate and high oleate but low stearate and arachidonate when compared with the phospholipids. In the fractionation of the neutral lipids by the method of Barron & Hanahan (1958), the triglycerides found were much less (72 $\mu$moles of triglyceride/g. of fat compared with 147 $\mu$moles of triglyceride/g. of fat). There was also less sterol ester (10·1 $\mu$moles/g. of fat compared with 33 $\mu$moles/g. of fat). This may be due to the younger age of the rats used in the second fractionation. The sterol fatty acids in the second extraction have a higher palmitoleate, stearate and arachidonate than in the fractionation described in Table 2. Oleate was the predominating acid in the sterol esters. The triglyceride fraction was unusual in having almost equal quantities of docosapentaenoate and docosahexaenoate (Table 4).

The non-polar phospholipid has a low amino nitrogen/phosphorus ratio (Table 3) and a very high content of unsaturated fatty acid, particularly linoleic acid (18-2). These analytical features are consistent with a fraction which contains a large amount of 'Cardiolipin' (Gray & Macfarlane, 1958; Getz & Bartley, 1959). The remaining phospholipids recovered from the second batch of rat livers were similar, on analysis, to those from the first batch and have therefore not been included in Tables 3 and 5. In this case, however, the lecithin fraction was analysed by the mild alkaline hydrolysis and paper-chromatography method of Dawson (1960); 96 % of the total phosphorus of the fraction was found as glycerylphosphorylcholine ($R_F$ 0·77 in phenol–acetic acid–ethanol–water solvent).

The phospholipids generally were low in oleate but contained large amounts of stearate and arachidonate, particularly in the inositide fraction. A high linoleate content was found in the triglyceride in the non-polar phospholipid fraction and in the sphingomyelin fraction.

The free fatty acid fraction (Table 3) was obtained from the triglyceride fraction by extraction with methanolic alkali (0·5 % of potassium carbonate in 50 % methanol) and subsequent esterification. The amount of free acid found was 6·55 $\mu$moles/g. dry wt. of liver. Table 3 shows that its composition differs somewhat from the triglycerides associated with it.

## DISCUSSION

The distribution of phosphorus between lecithin and other phospholipids reported in this paper accords well with that given by Hanahan *et al.* (1957), and with that tabulated by Dawson (1957) in his review of animal phospholipids. The mean value of 35·8 $\mu$moles of phospholipid/g. wet wt. of liver is to be compared with 39 $\mu$moles/g. wet wt. given by Artom (1945). Lecithin and sphingomyelin constituted 60 % (23·4 $\mu$moles/g. dry wt.) of the phospholipid. This corresponds closely with our values.

The fatty acid composition of the liver-lipid fractions is similar to those reported by Macfarlane, Gray & Wheeldon (1960) and Dittmer & Hanahan (1959). The latter authors used reversed-phase column chromatography for the separation of fatty acids and their sterol ester fraction has a higher stearate and arachidonate than ours. Dittmer & Hanahan did not estimate the minor fatty acids individually and this may account for the discrepancy. Generally, similar results have also been obtained with alkali isomerization for the estimation of unsaturated fatty acids of rat-liver cholesterol ester, triglyceride and phospholipid by Okey & Harris (1958), except that our values for dienoic acids and tetraenoic acids are somewhat lower in each case.

Studies of fatty acid composition of liver lipids have been made in other species. For instance, Bruce, Shorland & Jessop (1952) studied horse liver and found a partitioning of palmitic acid, stearic acid and unsaturated $C_{18}$ and $C_{20}$ acids between neutral glycerides and phospholipids that was broadly similar to that found in this study, except that the horse liver contained much more linolenic acid than did rat liver. Linolenic (18-3) acid was present in only very small amounts in the rat liver and especially in the triglycerides, although it was present in the diet (Getz & Bartley, 1961). Hilditch & Shorland (1937) have also reported a similar distribution of fatty acids between neutral glyceride and phospholipid fatty acids of the livers of the ox, cow, sheep and pig, with some small exceptions. The pig- and sheep-liver glycerides have higher stearate and arachidonate than is normally found in neutral glycerides, and the cow-liver phosphatide had a surprisingly small quantity of unsaturated $C_{20}$ acid.

In general, however, all studies including this one indicate the presence of high palmitate, and $C_{18}$ unsaturated acids with low stearate and $C_{20}$ unsaturated acids in neutral-lipid fractions, whereas phospholipids are characterized by relatively high stearate and $C_{20}$–$C_{22}$ polyunsaturated acids together with low $C_{18}$ unsaturated acids. There is, however, a considerable variation in the fatty acid composition of the individual phospholipids. This is in contrast with similarity of fatty acid composition within the cell organelle. Thus irrespective of the location within the cell, the phospholipid tends to have the same fatty acid composition.

## SUMMARY

1. The lipids of the rat liver have been fractionated into cholesterol esters, triglycerides, serine phosphatides, ethanolamine phosphatides, inositol phosphatides, lecithin and sphingomyelin, and the fatty acid content of the isolated lipids has been measured.

2. More than 50 % of the lipid phosphorus was found in the phosphatidylcholine fraction. Most of the neutral lipid was in the form of triglycerides.

3. The neutral lipids were high in palmitate and $C_{18}$ unsaturated fatty acids whereas the phospholipids had relatively high stearate and $C_{20}$–$C_{22}$ polyunsaturated fatty acids with low $C_{18}$ unsaturated acids.

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# The Mechanism of Fructose Formation in Goat Placenta with Special Reference to the Possible Involvement of Sorbitol or of Phosphoric Acid Esters

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The presence of fructose in the foetal blood of ungulates and its virtual absence from the maternal blood have been frequently observed (e.g. Goodwin, 1956). Huggett, Warren & Warren (1951) provided evidence, by means of both infusion and perfusion experiments, that the placenta was the site of fructose formation in the sheep. Walker (1960) used the infusion technique with the separation of one of twin foetuses to demonstrate that the placenta is a site of fructose formation from glucose in the goat also. Perfusion experiments are reported in the present paper which confirm this finding for the goat.

The mechanisms of fructose formation from glucose remains obscure. Two hypotheses to explain fructose formation are considered in this paper. One, put forward by Winterton (1949), suggested that fructose was formed in sheep placenta by the action of a phosphatase on a fructose phosphoric acid ester. The second hypothesis, that fructose might be formed from glucose via sorbitol, arose from the observation by Williams-Ashman & Banks (1954) that sorbitol dehydrogenase (ketose reductase) was present in the seminal vesicles of the rat and the suggestion that this could be part of a mechanism for the formation of fructose by this tissue. That fructose is indeed formed from glucose via sorbitol in sheep seminal vesicles has recently been convincingly demonstrated by Hers (1960*a*, *b*).

The results reported in this paper supply some evidence alongside which these hypotheses may be considered. A preliminary account of some of this work was given by Neil, Walker & Warren (1957).

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## EXPERIMENTAL

Details concerning goats, surgical procedure, infusion of sugars (and, in this paper, sorbitol), collection of blood samples, chromatography of blood extracts and the determination of sugars have been given by Walker (1960).

*Perfusion of the placenta.* A Dale–Schuster pump unit was employed. In the initial experiments heparinized maternal-goat plasma was used as perfusion fluid, but more satisfactory results were obtained later with a dextran–saline solution having the following composition: dextran, 5·1% (w/v); NaCl, 0·76%; $KHCO_3$, 0·24%; equilibrated with $O_2 + CO_2$ (95:5) at 37°. The umbilical-cord vessels were dissected and one vein and one artery cannulated. Perfusion into the artery was started, and the remaining pair of uncannulated vessels immediately ligatured. The foetus was then removed by cutting the cord. The perfusion fluid was sampled at intervals for sugar analyses.

*Detection of sorbitol-dehydrogenase activity.* Homogenates of placental tissue (10% in 0·25M-sucrose) or extracts of acetone-dried powders of placental tissue were assayed by the method of Blakley (1951) as described by Wolff (1955). The advisability of using whole homogenates where possible for detection of the enzyme was indicated by Blakley (1951). He found, using the Warburg technique, that extracts of rat kidney, for example, failed to oxidize sorbitol, whereas a whole homogenate of this tissue did so. In this work the Thunberg-tube technique was chosen as a rapid and simple method for detection of the enzyme with whole homogenates.

The tubes contained the following mixture: potassium phosphate buffer, 1·0 ml., 0·1M, pH 8·0; sorbitol, 0·2 ml., 0·1M; diphosphopyridine nucleotide (DPN), 0·1 ml., 5 mM; sodium malonate, 0·3 ml., 1·0M; nicotinamide, 0·3 ml., 1·0M; methylene blue, 0·1 or 0·3 ml., 1 mM; placental homogenate, 0·5 or 0·25 ml., 10% in water in most experiments; water to 3·0 ml. Control tubes contained water in place of sorbitol solution. The tubes were evacuated and incubated at 37°. Enzyme activity was expressed as the

ratio of the decolorization time of the control (C) to that of the assay solution (T).

Human placentas were obtained at parturition and analysed immediately. Pig placentas were obtained through the co-operation of T. Wall and Sons Ltd., were placed on ice immediately after removal from the animals, and assayed 2–3 hr. later. One sample of pig placenta was placed on ice immediately after being shed at parturition, and analysed 2–3 hr. later. Cow placenta was obtained similarly and analysed 24 hr. after being placed on ice. Horse placentas, obtained by the kind co-operation of Professor W. C. Miller, Equine Research Station, Newmarket, were analysed immediately after being shed at parturition. Samples of placentas from other species were obtained in the laboratory by laparotomy, and analysed immediately.

### *Analysis of phosphoric acid esters*

*Preparation of extracts of placenta.* Cotyledons were removed from the placenta by tying off and cutting on the cotyledon side of the ligature, so as to avoid haemorrhage. The samples were rapidly freed from connective tissue, blotted, and then plunged into liquid $N_2$. When frozen the material was crushed to a powder with a stainless-steel mortar and pestle. The powder was ground up with an equal weight of ice-cold $HClO_4$ (15%, w/v) and allowed to thaw. The mixture was centrifuged at 0° and the supernatant decanted. The residue was re-extracted twice with the same volume of ice-cold $HClO_4$ (7·5%, w/v) with centrifuging. All the supernatants were pooled and adjusted to pH 5·0 with carbonate-free NaOH. Fractions of the phosphorus-containing compounds in the placental extracts were prepared by slight modifications of the conventional barium acetate–ethanol precipitation procedures described by LePage (1951), with precautions to exclude $CO_2$. The precipitates were removed by centrifuging, washed first with ice-cold 80% ethanol, followed by absolute ethanol, and finally with ether. The resulting powders were dried *in vacuo* over $CaCl_2$. Samples were then extracted with ice-cold HCl (0·1 N) three times. The residue after extraction was practically phosphate free. The combined extracts were ultrafiltered at 0° through shells (Membranfiltergesellschaft, Göttingen) to remove mucoid material. The ultrafiltrate was neutralized and stored at −10° before column analysis.

*Paper ionophoresis.* This was carried out on extracts of placenta during preliminary experiments. The conditions used were: Whatman no. 4 paper, washed as recommended by Walker (1953); barbitone buffer, pH 8·6, *I* 0·05; 400 v, corresponding to approximately 0·4 mA/cm. width of paper; 3–4 hr. This method achieves a separation of phosphoric acid and its esters into groups (Neil & Walker, 1954).

*Detection of phosphoric acid and its esters on paper.* The methods available for detection of phosphoric acid and its esters on paper (e.g. Hanes & Isherwood, 1949; Wade & Morgan, 1953) have not proved entirely satisfactory in this Laboratory, especially when used for detection on buffered paper. The following method was therefore devised and has proved satisfactory for a large range of buffered papers.

After ionophoresis or chromatography the paper is dried and dipped in an acetone solution of $FeCl_3$ [0·025% $FeCl_3,5H_2O$ in acetone containing 0·25% (v/v) of 10 N-HCl]. The acetone is allowed to evaporate, and the paper then dipped in an acetone solution of αα′-dipyridyl (0·1%) and dried at room temperature. The paper is now exposed to a damp atmosphere containing $SO_2$. Generation of $SO_2$ in a tank containing a beaker of hot water has been found to be satisfactory. The phosphoric acid and its esters appear as white spots on a rose–pink background after about 10–30 sec. exposure in the tank. The paper is immediately removed, and the spots remain unchanged for several days. The resulting paper is suitable for photography and further chemical analysis of the phosphorus-containing areas. The sensitivity of the method is affected by the buffer present in the paper, but on unbuffered paper the sensitivity limit is about 0·5 μg. of P/cm.² with inorganic orthophosphate as test substance.

*Analysis of extracts by gradient elution from an ion-exchange column.* Placental extracts, prepared as above, were placed on a De-Acidite FF (borate form) column and developed by gradient elution according to the method described by Parr (1955). About 350 fractions (10 ml. each) were collected. Each fraction was assayed for total phosphorus by the method of Allen (1940). The phosphorus-containing peaks thus revealed were further analysed for acid-labile phosphoric acid derivatives (N-$H_2SO_4$, 100°, 10 min.); hexose esters were estimated as total hexose by the anthrone method (Trevelyan & Harrison, 1952); fructose esters as fructose by the method of Cole (as described by Bacon & Bell, 1948); pentose esters by the method of Brown (1946); purine and pyrimidine nucleotides by measurement of absorption at 260 mμ.

## RESULTS

### *Effects of injecting sorbitol into the maternal and foetal circulations*

Sorbitol was injected into both maternal- and foetal-goat circulations with a view to determining whether this hexitol can act in the same way as glucose in promoting fructose formation by the placenta. The sorbitol was injected in all cases as an aqueous 20% (w/v) solution previously warmed to 37°. All the figures quoted refer to the sugar concentrations in samples of whole blood.

*Injection of sorbitol into the maternal circulation.* Sorbitol (1·5 g./kg. maternal body wt.) was injected into the maternal circulation. Two experiments of this type were performed. Blood samples were taken before and at intervals over a period of several hours after the injection of sorbitol. The result of such an experiment is shown in Fig. 1. The maternal-blood fructose and glucose values started to rise immediately after sorbitol injection. The increases in concentration above the initial values were similar in both cases, but the peak in the fructose concentration was reached before that of glucose. Both peaks were reached within about 80 min. of sorbitol injection and both values returned slowly to normal after a further 2–2½ hr. That fructose and not sorbose was present in the maternal blood was confirmed by paper chromatography. The foetal-blood total reducing sugar rose slowly after the sorbitol injection. This rise was almost entirely due to a rise in the blood-fructose

value, and was greater than that which would have been expected had it been caused solely by the increase in maternal-blood glucose noted above. Since the maternal-blood fructose could not have entered the foetal blood (Walker, 1960), this result suggests that the sorbitol injected into the maternal circulation acted as a precursor of the fructose that appeared in the foetal blood. The result of this experiment when viewed in the light of the perfusion experiments described below suggests that the goat placenta is permeable to sorbitol and that the site of the fructose formation resulting in the increase in foetal-blood fructose observed in the present experiment was the foetus and not the placenta.

*Injection of sorbitol into the foetal circulation.* Sorbitol (approx. 1 g./kg. estimated foetal body wt.) was injected via the umbilical cord into the foetal circulation. Two experiments of this type were performed. Samples of maternal and foetal blood were analysed for total reducing sugar and fructose. The maternal-blood total reducing sugar rose slowly over a period of about 3 hr. after the sorbitol injection to a value about 50 % above the initial concentration (Fig. 2). The foetal-blood total reducing sugar rose steadily, after the injection, to a value about 100 % above the starting concentration. This rise was almost entirely due to an increase in the fructose concentration. Since this fructose cannot pass into the maternal circulation, the rise in the maternal total reducing sugar suggests that sorbitol passed across the placenta from foetus to mother. No increase in foetal-blood glucose occurred, a result in contrast with that observed in maternal blood when sorbitol was injected into the maternal circulation. It was found by Walker (1960) that the injection of fructose into the foetal circulation also failed to evoke a rise in foetal-blood glucose.

*Perfusion of the placenta*

The volume of the perfusion fluid was about 120 ml. and the perfusion of the placenta was continued for 4 hr. Sorbitol (1 g./kg. estimated foetal body wt.) was injected into the perfusion circulation after an initial control period of 2 hr. Samples of perfusion fluid were taken at intervals for total reducing sugar and fructose determinations. The results are shown in Fig. 3.

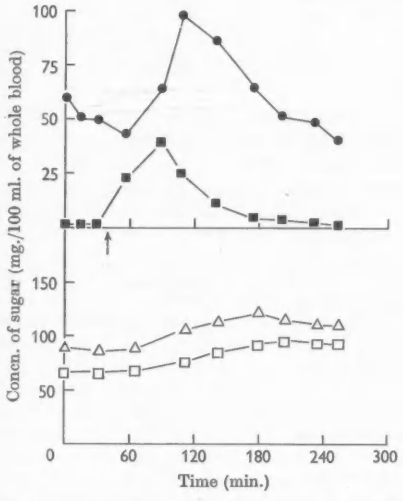


Fig. 1. Pregnant goat no. 40. Foetal age 138 days; singleton. Sorbitol (1·5 g./kg. maternal body wt.) was injected intravenously into the maternal circulation between 36 and 44 min. ●, Maternal glucose; ■, maternal fructose; △, foetal total reducing sugar; □, foetal fructose. The arrow indicates mean time of injection.

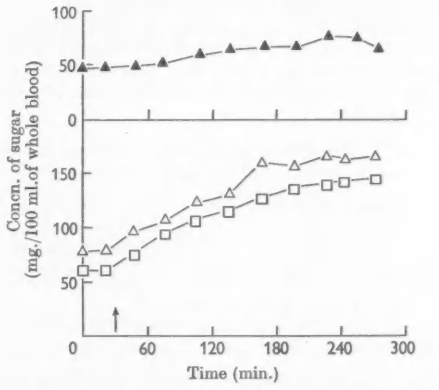


Fig. 2. Pregnant goat no. 42. Foetal age 134 days; singleton. Sorbitol (1 g./kg. foetal body wt.) was injected into the umbilical vein between 28 and 32 min. ▲, Maternal total reducing sugar; △, foetal total reducing sugar; □, foetal fructose. The arrow indicates mean time of injection.

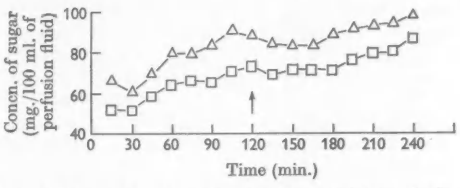


Fig. 3. Pregnant goat no. 48. Foetal age 135 days; singleton. Sorbitol (4 g. in 15 ml. of water) was injected into the perfusion fluid between 120 and 124 min. △, Perfusion-fluid total reducing sugar; □, perfusion-fluid fructose. The arrow indicates mean time of injection.

During the control period (i.e. up to 120 min.) the fructose concentration rose slowly to a value of about 75 mg./100 ml. of perfusion fluid. On injection of the strongly hyperosmotic sorbitol solution (15 ml.) at the 120th min., the volume of perfusion fluid increased slowly by a further 20 ml., owing to the passage of water from the maternal blood into the pump system. During this period of 1 hr. (i.e. up to the 180th min.) no increase in fructose concentration in the perfusion fluid was noted. Thereafter, over a period of about 1 hr., the fructose concentration slowly rose again at approximately the same rate as during the control period. There was no evidence during the period over which determinations were made of any marked increase in the rate of fructose formation due to the presence of sorbitol. The glucose concentration in the perfusion fluid remained fairly steady throughout the experiment. In all, three perfusions were carried out, with injection of sorbitol, and in no case was evidence obtained indicating that sorbitol influenced the rate of fructose formation by the placenta.

The rise in fructose concentration in the perfusion fluid during the control period supports the findings of Walker (1960) that, in the goat, as in the sheep, the placenta is a site of formation of foetal-blood fructose.

*Detection of sorbitol dehydrogenase in placentas*

Sorbitol-dehydrogenase activity was detected by the methods described above and the results are shown in Table 1. Some confirmatory experiments on sheep placenta were carried out on acetone-dried powders kindly supplied by Dr E. C. Long. The conditions for detecting the enzymic activity were varied considerably during the experiments on pig and horse placentas but every test was completely negative.

It is quite clear from Table 1 that the presence of the dehydrogenase cannot be correlated either with the fructose-forming potential of the placenta or with gross placental morphology.

*Phosphoric acid and its esters in goat-placental tissue before and after injection of glucose into the maternal circulation*

Preliminary analyses on perchloric acid or trichloroacetic acid extracts of placental tissue for inorganic orthophosphate and acid-labile and acid-stable phosphoric acid derivatives were carried out by the methods outlined above. The variability of the results (Table 2) suggested that such analyses would not be helpful in obtaining the information required. Further preliminary analyses were carried out by ionophoresis of solutions of fractions of the phosphorus-containing compounds which had been obtained from the extracts by barium acetate–ethanol precipitation. This procedure separated the phosphorus compounds present into four distinct groups. Quantitative analysis of these groups indicated that the amount of fructose 1:6-diphosphate in the placental extracts rose by a factor of two to three after the injection of glucose into the maternal circulation. Examination of an acid hydrolysate of the barium fraction by ionophoresis indicated the absence of phosphoric acid esters of sorbitol.

Table 1. *Placental type, principal foetal-blood sugar and placental tissue sorbitol-dehydrogenase activity in various species*

The enzyme was detected as described in the Experimental section, and activities are expressed in terms of the ratio (C/T) of the time taken for decolorization of the control solution to that for decolorization of the assay solution. + + +, indicates C/T > 15; +, C/T 4–9·9; −, indicates no activity detected.

| | Gross placental morphology (all chorioallantoic) | Sorbitol-dehydrogenase activity in placenta |
|---|---|---|
| | Principal foetal-blood sugar: fructose | |
| Cow | Polycotyledonary, villous | + |
| Goat | Polycotyledonary, villous | + + + |
| Sheep | Polycotyledonary, villous | + |
| Pig | Diffuse, villous | − |
| Horse | Diffuse, villous | − |
| | Principal foetal-blood sugar: glucose | |
| Man | Discoid, villous | − |
| Dog | Annular or zonary, labyrinthine | − |
| Cat | Annular, labyrinthine | − |
| Guinea pig | Subspheroid, labyrinthine | + + + |
| Rabbit | Discoid, labyrinthine | + + + |
| Hamster | Discoid, labyrinthine | + + + |
| Mouse | Discoid, labyrinthine | − |
| Multi-mammate rat | Discoid, labyrinthine | − |
| Jird (*Meriones libycus*) | Discoid, labyrinthine | − |
| Albino rat | Discoid, labyrinthine | − |

Table 2. *Analyses of acid extracts of goat placentas for inorganic orthophosphate and acid-labile* and acid-stable phosphoric acid derivatives*

Values given (mean ± S.D.) are phosphorus (P) as a percentage of the total acid-soluble phosphorus extracted. Cotyledons were removed for analysis immediately before sugar infusion into the maternal circulation (zero time) and at subsequent intervals as indicated.

| Sample time (min.) | No. of goats | Inorganic phosphate | Acid-labile* P derivatives | Acid-stable P derivatives |
|---|---|---|---|---|
| 0 | 6 | 24·6 ± 4·6 | 10·2 ± 3·3 | 65·2 ± 4·6 |
| 30 | 6 | 26·4 ± 6·2 | 8·3 ± 2·1 | 65·3 ± 5·4 |
| 60 | 5 | 26·9 ± 7·9 | 8·7 ± 2·4 | 64·4 ± 5·7 |
| 120 | 3 | 29·7 ± 5·4 | 6·4 ± 1·6 | 63·9 ± 4·7 |

* Acid-labile P derivatives are represented by the increase in inorganic P after 10 min. at 100° in N-$H_2SO_4$.

During the development of methods for separating the phosphorus-containing compounds from the perchloric acid extracts from cotyledons, it was observed that the amount of these compounds precipitated by the barium acetate–ethanol procedure was much increased if the sodium hydroxide used to neutralize the acid extracts contained appreciable amounts of carbonate. The extra phosphorus-containing material which was precipitated in these conditions, but which appeared only in very small amounts in precipitates obtained from carbonate-free solutions, comprised 50–70 % of the acid-soluble phosphorus compounds in the acid extracts; further, it consisted of an acid-stable phosphoric acid monoester fraction which contained practically no esterified hexose, pentose, glycerol or glyceric acid. Qualitative tests indicated the presence of esterified choline in the fraction and a more detailed analysis of the compounds present is presented in the addendum to this paper.

Extracts were prepared, as described in the Experimental section, of placental tissue taken before, ½ hr. after and 2 hr. after the injection of glucose into the maternal circulation. Quantitative analyses of the three ultrafiltrates prepared from these extracts were carried out with the gradient elution method, and eleven peaks containing phosphoric acid and its esters were observed in each of the three analyses. Each of the phosphate-containing fractions was present in each sample analysis and there were no additional peaks.

Most of the fractions were identified by using the special colour reactions indicated in the Experimental section and by paper chromatography. Over 98 % of the second elution peak was inorganic orthophosphate and 75–80 % of the ninth peak proved to be fructose 1:6-diphosphate. Quantitative comparison of the elution patterns obtained from the cotyledons taken at the three different times showed that the only significant changes in the distribution of phosphorus between the elution fractions occurred in the inorganic orthophosphate and the fructose 1:6-diphosphate peaks. Half an hour after the injection of glucose into the maternal circulation the percentage of phosphorus present as fructose 1:6-diphosphate in the placental tissue extract had risen from 2·4 to 7·6, while that present as inorganic orthophosphate had fallen from 66·8 to 56·3. This pattern of altered P distribution was still apparent 2 hr. after glucose injection but the percentages observed (6·2 and 59·6 respectively) indicated a slow return towards the control values.

## DISCUSSION

Walker (1960) demonstrated that the placenta of the goat was freely permeable in both directions to glucose, galactose, mannose and xylose, but not to fructose or sorbose or to the disaccharides sucrose, maltose and lactose. The effects of sorbitol injected into the maternal and foetal circulations of the goat on the foetal- and maternal-blood sugar concentrations respectively, as reported in this paper, suggest that the placenta is also permeable to sorbitol in both directions. The present experiments do not give any direct measure of the rate of sorbitol transport across the placental barrier, although it is probably much less rapid than that of glucose. The rise in foetal fructose caused by an injection of sorbitol into the maternal circulation is only a fraction of that caused by a similar injection of glucose. This could be explained by the much slower transport of sorbitol across the placenta than is the case with glucose. The rise in foetal fructose on injection of sorbitol into the foetal circulation is greater and more prolonged than that produced by glucose injection. Sorbitol in the foetal circulation is readily converted into fructose, presumably by the action of sorbitol dehydrogenase present in the foetal liver (Hers, 1960*b*). These observations, together with the absence of enhanced fructose formation by the placenta perfused with dextran–saline containing sorbitol, suggest that fructose formation from sorbitol does not occur in goat placenta.

Injection of sorbitol into the maternal circulation leads to the appearance of fructose in the maternal

blood and to an increase in the glucose concentration. This additional glucose is probably formed from fructose since the latter reaches its maximum concentration first. However, neither sorbitol nor fructose injected into the foetal circulation gives rise to an increase in foetal-blood glucose concentration [this paper and Walker, (1960)]. This constitutes an interesting difference between the metabolism of these compounds in adult and foetal tissues.

Perfusion of the goat placenta [and also of sheep placenta; (Long, 1957)] did not enhance the rate of increase of fructose concentration in the perfusion fluid. These findings are perhaps surprising in view of the sorbitol-dehydrogenase activities demonstrated in these tissues *in vitro* (Table 1) but may conceivably be attributable to failure of the sorbitol, in the perfusion fluid, to penetrate to the site of the placental enzyme. Wick & Drury (1951) reported such a permeability difference between glucose and sorbitol in rabbit muscle. Hers (1960*b*), using an assay system in which DPN was not added, found that sorbitol was rapidly converted into fructose by foetal-sheep liver but only slowly, if at all, by sheep placenta. The latter observation is in agreement with the results of the perfusion experiments described above. Hers (1960*b*) also demonstrated the presence of an active aldose reductase in both sheep and cow placentas, and suggested that reduction of glucose to sorbitol occurs in the placenta and that the sorbitol is converted into fructose in the foetal liver. Nevertheless, the perfusion experiments described in the present paper demonstrate that fructose is formed by the goat placenta [as it is in the sheep (Huggett *et al.* (1951)] in the absence of the foetus. This suggests that foetal-blood fructose in these species may arise by more than one mechanism.

Although sorbitol dehydrogenase was shown to be present in placental tissue from goat, cow and sheep, attempts to demonstrate its presence in the placental tissues from pig and horse (which also have fructose as the main foetal-blood sugar) failed. The presence of sorbitol dehydrogenase in the placentas from non-ungulates and the absence of appreciable amounts of fructose in the foetal blood of such species strengthens the conclusion that the occurrence of this enzyme in mammalian placentas is not associated with the formation of foetal-blood fructose.

If phosphoric acid esters, rather than sorbitol, are involved in the placental conversion of glucose into fructose it seemed reasonable to suppose that the administration of large amounts of glucose might evoke changes in the concentrations of phosphoric acid esters indicative of the mechanism of conversion. The experiments reported here show that, in goat placenta, only fructose 1:6-diphosphate accumulates to any marked extent when glucose is injected into the maternal circulation. The accumulation of fructose 1:6-diphosphate in particular may merely reflect enhanced glucose metabolism under the experimental conditions. Dempsey & Wislocki (1947), using histochemical techniques, showed that fructose 1:6-diphosphatase activity was present in placental tissue from the guinea pig, cat, sow and human. However, their results did not suggest that notably high activity, or marked specificity, of the enzyme responsible could be associated particularly with ungulate placentas.

Therefore, although the accumulation of fructose 1:6-diphosphate in the experiments described here may well be a reflexion of the mechanism for the formation of foetal-blood fructose by goat placenta, more specific evidence for this view is at present lacking.

## SUMMARY

1. Experiments designed to examine current hypotheses concerning the mechanism of fructose formation from glucose in ungulate placentas have been carried out.

2. The goat placenta is permeable to sorbitol. Injection of sorbitol into the maternal circulation increases the concentration of both fructose and glucose in the maternal blood, but injection into the foetal circulation increases only the foetal-blood fructose.

3. In the absence of the foetus, perfusion *in situ* of the foetal side of the goat placenta with sorbitol does not increase the rate of fructose formation.

4. The presence or absence of sorbitol dehydrogenase (ketose reductase) in the placentas of 15 species of animals cannot be correlated with the nature of the principal foetal-blood sugar.

5. Injection of glucose into the maternal blood leads to a marked increase in fructose 1:6-diphosphate in the placenta and to a decrease in placental inorganic orthophosphate.

6. Current hypotheses of fructose formation from glucose in ungulate placenta are reviewed in the light of these findings.

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## ADDENDUM

# The Occurrence of Phosphorylcholine and Phosphorylethanolamine in Goat Placenta

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In the main part of this paper it was mentioned that of the phosphorus-containing compounds obtained from goat cotyledons by acid extraction followed by precipitation as barium salts in the presence of ethanol (fraction A; preparation described below) between 50 and 70% consisted of an acid-stable phosphoric acid monoester fraction that contained practically no esterified hexose, pentose, glycerol or glyceric acid. Further characterization of this fraction is presented as an addendum for two reasons: (1) The analyses reported here were carried out on a single specimen of material obtained by pooling fractions A obtained from cotyledons from two goats. (2) The specimen analysed represents material isolated in the first instance for hexose phosphate analyses, and the method of isolation is not necessarily optimum for quantitative estimation of phosphorylcholine and phosphorylethanolamine in goat-placental tissue.

Preliminary electrophoresis of fraction A revealed that a group of the phosphoric acid derivatives present, representing 59·1% of the total phosphorus in the material, could be separated from the hexose phosphoric acid derivatives and inorganic phosphate. This group was slow-moving relative to inorganic orthophosphate and bore a net negative charge at pH 8·6 in barbitone buffer; it contained nitrogen, and preliminary qualitative analysis indicated the presence of esterified choline. Material having similar properties was prepared by fractionation of fraction A on De-Acidite FF ion-exchange resin (see below); the material so treated is referred to as fraction B.

As considerable amounts of phosphorylcholine and phosphorylethanolamine occur in rabbit uterus, and perhaps also in human, cow and pig uteri (Cawkwell, 1958), it seemed of interest to investigate further the goat-placental fraction B. The results presented here indicate that phosphorylcholine and phosphorylethanolamine together account for about four-fifths of the esterified phosphorus in the fraction. A preliminary account of this work was given by Neil (1960).

## MATERIALS AND METHODS

*Preparation of fraction A.* The $HClO_4$ extract of cotyledons was adjusted to pH 4 with bench 10N-NaOH (which was about 0·1N with respect to $Na_2CO_3$), 4 vol. of ethanol (95%, v/v, pH 4) were added and the solution was kept at 2° for 18 hr. The precipitate was removed by centrifuging and NaOH added to the supernatant solution until it was just pink to phenolphthalein. An excess of barium acetate (25%, w/v) was added and the ethanol content adjusted to about 80% (v/v), after which the solution was kept at 2° for 18 hr. in a vessel open to the atmosphere. The material that was precipitated during this period was removed by centrifuging at 2° and washed twice with ice-cold ethanol [80% (v/v) adjusted with NaOH to be just pink to phenolphthalein], twice with absolute ethanol, twice with dry ether and placed in a vacuum desiccator over $CaCl_2$.

*Preparation of fraction B.* The dry fraction A prepared as above was suspended in water and dissolved by addition of a minimal quantity of HCl; it was then freed from barium by passage through a column of Zeo-Karb 225 ($H^+$ form). The effluent was passed through a De-Acidite FF ($Cl^-$ form) column and washed through with water. The eluate comprised fraction B, together with $Cl^-$, and contained 71·4%

of the total phosphorus present in fraction A. The eluate was freed from $Cl^-$ by successive passage through two columns of Zeo-Karb 225, the first being in the $Ag^+$ form and the second in the $Na^+$ form. The $Cl^-$-free eluate was concentrated by distillation under reduced pressure at a temperature not exceeding 40°. The final concentrate of fraction B contained 27·6 μg.atoms of P/ml.

*Hydrolysis of fraction B.* Phosphomonoesterase hydrolyses of fraction B were carried out at 37° in carbonate–bicarbonate buffer, pH 10, until there was no further increase in the inorganic phosphate content of the solution even upon further addition of enzyme. Controls were carried out with boiled enzyme.

*Chromatography.* Fraction B was fractionated on chromatograms run for 24 hr. on Whatman no. 1 paper, the mobile phase being 80% (w/v) phenol in water, in an atmosphere of $NH_3$. The phosphoric acid esters were detected by the αα′-dipyridyl method described in the main part of this paper. Parallel chromatograms were dipped in a solution of 0·2% ninhydrin in 95% (v/v) acetone. Chromatography of dinitrophenyl derivatives was carried out with the butanol–water system of Mellon, Korn & Hoover (1953).

*Quantitative analysis.* Phosphorus as inorganic orthophosphate was estimated either by the method of Allen (1940) or by that of Fiske & Subbarow (1925). Choline was assayed by the periodide method of Appleton, LaDu, Levy, Steele & Brodie (1953). Ethanolamine was estimated by the fluorodinitrobenzene method of Axelrod, Reichenthal & Brodie (1953).

*Differential hydrolyses.* Estimations of glycerylphosphorylethanolamine and phosphorylethanolamine and of glycerylphosphorylcholine and phosphorylcholine in admixtures were carried out by differential hydrolyses as described by Cawkwell (1958), followed by assays of the free bases liberated.

*Materials.* Phosphorylcholine was prepared by the method of Riley as described by Schmidt (1957). Dinitrophenylethanolamine was a gift from Dr J. S. Fawcett. Dinitrophenylserine was prepared by an adaptation of the method of estimation of serine (Axelrod *et al.* 1953). Phosphomonoesterase was obtained from the Sigma Chemical Co., St Louis 18, Mo., U.S.A., and was virtually free from diesterase activity. A second sample of phosphomonoesterase having negligible diesterase activity was kindly supplied by Dr P. Hele.

## RESULTS

Paper chromatography of fraction B in phenol–ammonia revealed four principal phosphorus-containing areas which had $R_F$ values of 0 (6%), 0·16 (10%), 0·35 (8%) and 0·86 (68%). Two of these areas ($R_F$ 0·16 and 0·35) also gave positive reactions with ninhydrin. The phosphorus contents of these areas (indicated in parentheses above as a percentage of the total phosphorus in the extract applied to the paper) and also of the intervening areas were determined by a conventional ashing technique. The phosphorus recovered in these analyses ranged from 92 to 97% of that applied to the start-line. The $R_F$ values of 0·35 and 0·86 corresponded to those of phosphorylethanolamine and phosphorylcholine respectively. Glycerylphosphorylethanolamine has an $R_F$ of 0·63 in this system and was absent from the extract. Glycerylphosphorylcholine is not clearly separated from phosphorylcholine in this system but its absence from the extract was indicated by the differential hydrolysis experiments described below. The phosphorus-containing material that remained on the start-line represents relatively non-specific adsorption since unwashed paper was used (Walker, 1953). The nature of the material of $R_F$ 0·16 was not determined.

*Choline analyses.* The absence of glycerylphosphorylcholine and presence of phosphorylcholine in fraction B is indicated by the results of differential-hydrolysis experiments. Hydrolysis by N-hydrochloric acid for 30 min. at 100° (which liberates free choline from glycerylphosphorylcholine but not from phosphorylcholine) liberated no free choline from fraction B. Hydrolysis by 2N-hydrochloric acid for 24 hr. at 123° (which liberates choline from both the mono- and the di-esters) liberated choline equivalent to 69·9% of the total phosphorus present in fraction B. The agreement between the results obtained by chromatography and by hydrolysis is reasonably satisfactory. Qualitative evidence confirming the presence of free choline after long hydrolysis of fraction B was provided by preparation of choline reineckate from the hydrolysate and by pyrolytic cleavage (Feigl, 1956). Further quantitative evidence, obtained by electrophoresis, confirming the analytical data is given below.

*Ethanolamine analyses.* The presence of phosphorylethanolamine in fraction B was suggested by preliminary electrophoresis experiments and by chromatography as described above. Estimation of free ethanolamine and serine by the fluorodinitrobenzene method (Axelrod *et al.* 1953), after acid hydrolysis of fraction B, did not prove satisfactory because the solvent extraction procedure is not very specific and because fraction B as prepared above is contaminated by small amounts of impurities that, after acid hydrolysis, yield dinitrophenyl derivatives that are extracted into both the chloroform and the *iso*butyl methyl ketone solvents. Hydrolysis by pure phosphomonoesterase in carbonate–bicarbonate buffer avoided these artifacts for ethanolamine determination. The results of ethanolamine estimations before and after hydrolysis by phosphomonoesterase revealed that this process liberated ethanolamine equivalent to 10·4% of the total phosphorus present in fraction B. The agreement between this result and that obtained by chromatography is not entirely satisfactory. The extent of recovery and the non-specific adsorption of material on the start-line mentioned above would be reflected by a much larger percentage error in the analyses of the relatively small amount of phosphorylethanolamine than in those for the large amount of phosphorylcholine. The figures obtained from

the chromatograms, therefore, are likely to be minimal.

Evidence that ethanolamine was liberated by the action of phosphomonoesterase was obtained by paper chromatography of the dinitrophenyl derivatives prepared from the hydrolysate. Only one derivative appeared in the hydrolysate but not in the control and it behaved just like pure dinitrophenylethanolamine in the butanol–water system of Mellon *et al.* (1953). Dinitrophenylserine was absent from both preparations.

*Electrophoresis.* Electrophoresis (barbitone buffer, pH 8·6, *I* 0·05, 0·4 mA/cm. width, Whatman no. 4 paper) of fraction A prepared as above from goat placenta revealed the presence of a phosphoric acid ester fraction that moved towards the anode at a rate of about 0·46 relative to inorganic orthophosphate (1·0). Most of the phosphorus-containing material in fraction B moved at the same rate as did a mixture of phosphorylethanolamine and phosphorylcholine under the same conditions. Determination of the total phosphorus on the paper after electrophoresis showed that 59·1% of the total phosphorus present in fraction A from goat placenta was present in the region of relative rate 0·46. Since fraction B accounts for 71·4% of the total phosphorus in fraction A, and phosphorylcholine and phosphorylethanolamine account for 80·3% of the total phosphorus in fraction B, the two esters account for 57·4% of the total phosphorus in fraction A. This figure is compatible with that obtained by quantitative electrophoresis described above.

## DISCUSSION

It is reasonable to conclude from these results that phosphorylcholine and phosphorylethanolamine are quantitatively important constituents of the phosphoric acid derivatives extractable from goat placenta by perchloric acid. From the nature of the extract analysed, as outlined in the introduction to this addendum, it is clear that the absence of the glyceryl derivatives of phosphorylcholine and phosphorylethanolamine does not exclude their presence in goat-placental tissue. All four of these derivatives have been shown to occur in human placenta (Porcellati, Curti & Luciani, 1959*a*, *b*).

## SUMMARY

1. Phosphorylcholine and phosphorylethanolamine are shown to be quantitatively important constituents of the phosphoric acid derivatives present in goat placenta.

I wish to thank Mrs Marian Horner for expert technical assistance.

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# The Electrophoresis of Histones and Histone Fractions on Starch Gel

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Histones can be divided into a number of fractions by the procedures of extraction, electrophoresis, sedimentation or chromatography (for review, see Butler, 1959). Chromatography on carboxymethylcellulose, as developed by Johns, Phillips, Simson & Butler (1960), gave three well-characterized fractions, a lysine-rich fraction (F1), a moderately lysine-rich fraction (F2) and an arginine-rich fraction (F3). Two of these, F2 and F3, are reasonably homogeneous with respect to their *N*-terminal groups.

These fractions have now been studied by the method of electrophoresis in starch gel introduced by Smithies (1955). Neelin & Connell (1959) re-

ported the fractionation in starch gel of chicken-erythrocyte histone into 16 bands at pH 4·1 and 4·9. They confirmed that these bands were not artifacts peculiar to the method by submitting them to a second electrophoresis, when the bands retained their homogeneity and relative mobilities. Neelin & Neelin (1960) further reported the separation of calf-thymus histone into 18 bands at pH 4·9 and 15 bands at pH 3·9, and by submitting these fractions to a second electrophoresis they again confirmed the consistency of the resolution, and substantiated the complexity of histone.

Sautière (1959), using gel at pH 4·1, also examined two fractions obtained from carboxymethylcellulose columns and found at least 14 bands in each case. The possibility of some aggregation occurring and giving extra bands at pH 4 must, however, be considered. For this reason, the experiments reported below have been confined to the separation of histone fractions at approx. pH 2.

## EXPERIMENTAL

*Preparation of whole histone.* The calf-thymus histone used for these experiments was prepared by the method of Davison, James, Shooter & Butler (1954), with the following modifications. The nucleoprotein was isolated by washing with acidified NaCl (Phillips & Johns, 1959) and extracted three times with twice its volume of 0·25 N-HCl. After clarification the histone was dialysed against 1 mN-HCl and freeze-dried. The analysis of the whole histone is given in Table 1.

*Preparation of histone fractions.* The histone fractions were prepared by column chromatography on carboxymethylcellulose (Johns *et al.* 1960) and isolated by precipitation with trichloroacetic acid at a final concentration of 0·3 M, or 0·9 M with the lysine-rich fraction F1. The precipitates were dissolved in 40 ml. of ethanol and 0·5 ml. of conc. HCl was then added, followed by 100 ml. of acetone. The precipitates were washed in ether and dried under vacuum. The white precipitates were all readily soluble in water to give clear solutions. The analyses of these fractions are given in Table 1.

*Amino acid analyses.* Amino acids and *N*-terminal groups were determined as described by Phillips (1958) and Phillips & Johns (1959).

*Starch-gel electrophoresis.* The method used was essentially the same as that described by Smithies (1955). Unbuffered 0·01 N-HCl was used for filling the electrode vessels and for making the gels. This gave the lowest possible conductivity at pH 2 and so minimized the heating effects. Platinum electrodes were used in conjunction with 2·5 l. capacity electrode vessels. The 0·01 N-HCl was renewed after each run.

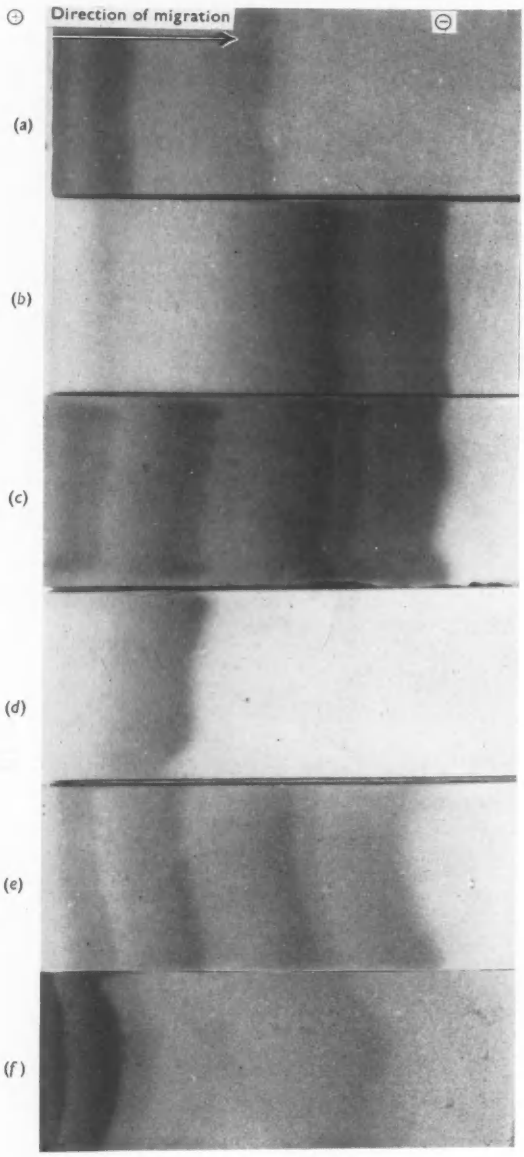
The pH of the gel was 2·3, and at this pH the resistance of a gel of dimensions 2 cm. × 0·5 cm. × 26 cm. was only 12 000 ohms. This low resistance limited the potential which could be applied to 100 v, because the heating became excessive at about 1 w. The sample (1·5 mg. of whole histone and 0·5 mg. of the fractions) was applied in 0·1 ml. of 0·01 N-HCl with starch powder as a supporting medium. No adsorption of histone to the starch grains was observed on staining the gel strips after the runs. The voltage gradient was 4 v/cm. and was applied for 18 hr. All runs were carried out at room temperature.

The histone in the gel was located by staining with naphthalene black. Permanent laboratory records were made by the contact-printing method of Johns (1960). These contact prints do not reproduce satisfactorily and for this purpose are replaced by photographs of the stained gel (Pl. 1) and by drawings showing the position of the bands (Fig. 1).

Table 1. *Composition of histone and histone fractions*

Amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. The proportions of *N*-terminal groups are molar percentages of all such groups found.

| | | Column fractions | | | Groups extracted from gel | | | |
|---|---|---|---|---|---|---|---|---|
| Amino acid | Whole histone | Arginine-rich (F3) | Lysine-rich (F1) | Slightly lysine-rich (F2) | E3 | E2 | E1 | Arginine-rich (extraction method) |
| Aspartic acid | 5·1 | 16·9 | 8·0 | 14·3 | 19·2 | 3·5 | 6·9 | 4·2 |
| Glutamic acid | 8·5 | | | | | 5·6 | 7·4 | 12·0 |
| Glycine | 7·9 | 6·6 | 7·2 | 10·3 | 6·4 | 6·8 | 9·7 | 6·1 |
| Alanine | 15·0 | 13·0 | 23·6 | 11·1 | 12·5 | 20·5 | 11·5 | 13·7 |
| Valine | 6·5 | 5·2 | 4·8 | 7·5 | 5·3 | 4·8 | 6·9 | 5·7 |
| Leucine + isoleucine | 11·5 | 13·8 | 5·4 | 12·7 | 10·0 | 7·2 | 11·6 | 13·6 |
| Phenylalanine | 1·7 | 2·9 | 0·7 | 1·5 | 3·2 | 0·9 | 1·7 | 2·4 |
| Tyrosine | — | 0·5 | 0·5 | 2·7 | — | 1·2 | 2·3 | 1·3 |
| Serine | 5·7 | 4·4 | 6·1 | 5·2 | 6·8 | 6·6 | 5·9 | 4·2 |
| Threonine | 6·0 | 6·2 | 5·6 | 5·4 | 6·1 | 6·0 | 5·9 | 6·9 |
| Proline | 5·6 | 4·9 | 8·9 | 3·6 | 5·3 | 8·1 | 4·3 | 4·7 |
| Histidine | 1·8 | 2·2 | 0·4 | 2·7 | 4·3 | 1·5 | 3·0 | 2·2 |
| Lysine | 16·4 | 10·3 | 25·9 | 13·1 | 11·0 | 23·1 | 13·3 | 9·0 |
| Arginine | 8·4 | 13·2 | 2·9 | 9·8 | 10·0 | 4·1 | 9·7 | 14·0 |
| Lysine/arginine | 2·0 | 0·78 | 8·9 | 1·34 | 1·1 | 5·7 | 1·38 | 0·64 |
| *N*-Terminal groups | | | | | | | | |
| Alanine | 39 | 69 | — | 10 | — | — | — | 96 |
| Proline | 49 | 14 | — | 70 | — | — | — | 3 |
| Others | 12 | 17 | — | 20 | — | — | — | 1 |




Starch-gel-electrophoresis patterns of whole histone and histone chromatographic fractions. (*a*) Arginine-rich histone F3; (*b*) slightly lysine-rich histone F2; (*c*) whole histone; (*d*) lysine-rich histone F1; (*e*) F1, F2 and F3 recombined; (*f*) arginine-rich histone made by the ethanol–HCl extraction procedure.

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## RESULTS

Some preliminary runs were carried out with whole histone in which the concentrations of acid in the gel and in the applied histone solutions were varied. The concentration of the acid in the gel is critical, as can be seen from Fig. 1*a*. In 0·01 N-HCl resolution into many bands occurred, but in 0·02 N-HCl only two bands appeared. Increasing the acid concentration in the applied histone solutions had no effect on the resolution of the bands but did slightly increase their mobilities (see Fig. 1*b*). In all subsequent runs 0·01 N-HCl was used for making the gel and for the sample application.

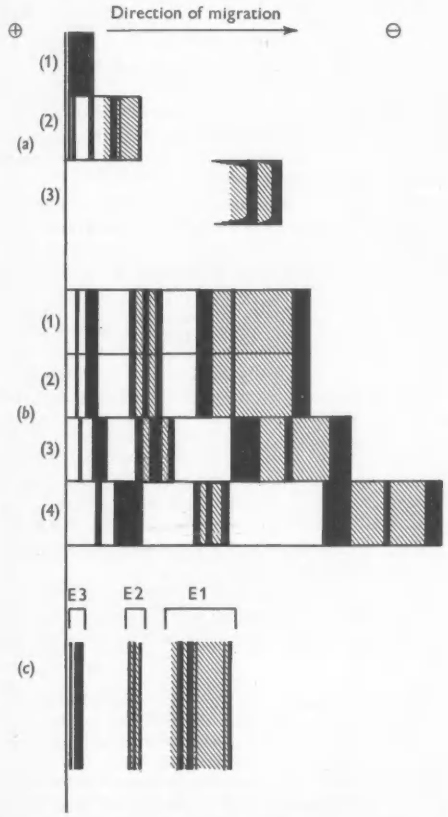


Fig. 1. (*a*) Starch-gel-electrophoresis patterns of whole histone, showing the effect of varying the acid concentration in the gel: (1) 1 mN-HCl; (2) 0·01 N-HCl; (3) 0·02 N-HCl. (*b*) Starch-gel-electrophoresis patterns of whole histone, showing the effect of varying the acid concentration of the applied solutions. The concentration of acid in the gel was 0·01 N in all experiments. The run lasted for 50 hr. instead of the usual 18 hr. (1) Water; (2) 0·01 N-HCl; (3) 0·1 N-HCl; (4) N-HCl. (*c*) Detailed pattern of whole histone, showing the subgroups. A wider tray, measuring 6 cm. × 0·5 cm. × 25 cm., was used for this experiment.

Under these conditions whole histone gave rise to at least 10 bands, falling into three main groups designated E1, E2, E3 in order of decreasing mobility, as shown in Fig. 1*c*. Group E1 had five distinct bands, group E2 at least three bands and group E3 two bands. Similar patterns have been obtained from four different preparations of the whole histone of calf thymus.

The histone fractions prepared by the methods described above have been submitted to electrophoresis under the same conditions and compared with the original histone (see Pl. 1). It can be seen that the arginine-rich fraction F3 gives rise to the electrophoretic group E3 of the whole histone; the moderately lysine-rich fraction F2 corresponds to group E1 and the lysine-rich fraction F1 corresponds to group E2. The fraction F3 used in this case was slightly contaminated with other proteins, possibly by degradation products, and the faint band with the higher mobility was not apparent in other arginine-rich fractions which were run.

The three histone chromatographic fractions were mixed together in the same proportions as they were obtained from the column and submitted to electrophoresis. The pattern obtained (see Pl. 1) was that of the original histone. Some attempts have been made to extract the histones of the different groups from the starch gel. The three groups from a run with whole histone were isolated by cutting the gel into portions and then submitting them to the freezing and thawing method described by Smithies (1955), 0·1 N-HCl being used as the solvent. A maximum of only 30 % of the histone present was recovered in this way. These protein solutions were hydrolysed in 6 N-HCl at 110°, and analysed in the usual way. If charring occurred owing to the residual starch in solution, a considerable loss of arginine and smaller losses of other amino acids were observed. Although no visible charring occurred in the analyses reported, it is still possible that the low arginine figure for E3 is due to this cause.

## DISCUSSION

The experiments described above show that the recombined chromatographic fractions gave the same electrophoretic pattern as the unfractionated histone. This demonstrates that extra bands have not been produced by the electrophoresis or by changes during chromatography. On the other hand, it is clearly indicated that all the fractions obtained from the column are complex.

Table 2. *Correlation of histone fractions obtained by chromatography and electrophoresis*

| | Lysine-rich | Slightly lysine-rich | Arginine-rich |
|---|---|---|---|
| Chromatographic fraction | F1 | F2 | F3 |
| Lysine/arginine | 8·9 | 1·34 | 0·78 |
| Electrophoretic groups (starch gel) | E2 | E1 | E3 |
| *N*-Terminal groups | — | Mainly proline | Mainly alanine |
| Other characteristics | High proline content | High glycine content | High acidic amino acids |
| | Low acidic amino acids | — | — |
| Fractions of Cruft, Hindley, Mauritzen & Stedman (1957) | $\alpha$ | $\gamma$ (?) | $\beta$ |

The arginine-rich fraction F3 (electrophoretic group E3, the slowest migrating group of bands) contains at least two proteins. The fraction used for these experiments had 69% of alanine *N*-terminal groups (Table 1, column 3), but other arginine-rich fractions prepared by an extraction procedure (Johns *et al.* 1960) having up to 96% of alanine *N*-terminal groups (Table 1, column 9) give the same two major bands on electrophoresis (Pl. 1*f*). It seems likely therefore that these two major constituents of group E3 both have alanine as their *N*-terminal group. The lysine/arginine ratio of the proteins extracted from the gel is higher than that of F3. This may be due to some loss of arginine on hydrolysis, or to fractionation during extraction from the gel. However, another definite characteristic of the arginine-rich histones, namely, the high content of acidic amino acids, is apparent in the analysis (Table 1).

The lysine-rich fraction (F1), which corresponds to electrophoretic group E2, gives a pattern of three bands, which may correspond to the three $\alpha$-fractions of Cruft, Hindley, Mauritzen & Stedman (1957). The analysis of this group, extracted from a gel after electrophoresis of the whole histone, gave 25·5% of lysine, thus confirming group E2 as the lysine-rich histone. Other confirmation comes from the high proline content and low content of acidic amino acids and leucine and isoleucine which are characteristic of this group (see Table 1).

The slightly lysine-rich fraction (F2) from the carboxymethylcellulose column, which has proline as its main *N*-terminal group, corresponds to electrophoretic group E1. One characteristic of this fraction is the very high glycine content of nearly 10%. It can be seen from Table 1 that group E1 is the only group with such a high value.

Groups E2 and E3 correspond both in order of electrophoretic mobilities and approximately in composition to electrophoretic fractions $\alpha$ and $\beta$ obtained by Cruft, Mauritzen & Stedman (1957) and Cruft *et al.* (1957). The fractions labelled $\gamma$ by Cruft *et al.* are possibly included in our group E1, but this cannot be decided until the subgroups can be isolated and analysed. The various correlations are given in Table 2.

Starch-gel electrophoresis, although a powerful means of resolution of histones, does not give patterns which are directly comparable with those obtained on free electrophoresis. It is possible, however, that a reversible type of adsorption is taking place and that the process is in fact electrochromatography (Svensson, 1956).

## SUMMARY

1. Electrophoretic patterns on starch gel at pH 2·3 have been obtained from whole calf-thymus histone and from the three fractions obtained by chromatography on carboxymethylcellulose.

2. The recombined chromatographic fractions, when run on starch gel, produced the original pattern of whole histone.

3. Correlations have been made between the chromatographic fractions and the electrophoretic groups, and other characteristics of these groups are given.

We wish to thank Mr D. Power for technical assistance. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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# Chromatographic Methods for the Study of Amines from Biological Material

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Amines occur in most biological fluids and extracts, and considerable work has been done on their isolation, in particular where the amines are of physiological interest, as are the catechol group, and 5-hydroxytryptamine and histamine. The methods used have usually been designed specifically for isolating the amine or group of amines in question, rather than for general application to all amines. The procedures presented here were chosen for their ability to deal with bases in general and for their relative mildness; they are not applicable to amino acids, which have already received adequate attention.

Since biological material usually contains amines in relatively low concentration, chromatography has been widely used to study them. Some of the methods have been adapted from those developed for amino acids; others have been worked out specially for amines (on paper: Bremner & Kenten, 1951; Schwyzer, 1952; Herbst, Keister & Weaver, 1958; on ion-exchange resins: Weber, 1951; Gardell, 1953; Kirshner & Goodall, 1957). Not all of the standard amino acid techniques can be applied to amines: thus electrolytic desalting only works with neutral compounds or ampholytes, and sulphonated polystyrene resins retain amines too firmly for convenient separation, so that rather powerful eluents are needed (Wall, 1953). Weakly acidic resins proved to have the necessary properties to overcome both these difficulties.

In the present work the amines were concentrated from a dilute solution or extract by ion-exchange resins used in the sequence described by Weber (1951), and the mixture of bases, after separation from most of the inorganic material, was resolved by column chromatography on buffered, weakly acidic resin. The fractions were analysed for amines, and those containing the peaks were then desalted on another column of resin in the free acid form. The resulting solution on evaporation to dryness yielded the crystalline amine hydrochlorides. Paper chromatography in five different solvents and paper electrophoresis were used to compare the products thus isolated with known compounds.

## MATERIALS AND METHODS

*Buffer solutions.* Except where otherwise stated, buffer solutions are those of McIlvaine (1921).

*Ion-exchange resins.* De-Acidite FFX (100-mesh beads, chloride form; Permutit Co. Ltd.). This resin was specially made for the present work, and differs from the commercially available De-Acidite FF by improved chemical stability. It was regenerated by stirring with twice its volume of 5% NaOH, filtering on a sintered funnel, and repeated washing on the filter with 5% NaOH until the filtrate was free of chloride. The resin was then washed with boiled (i.e. $CO_2$-free) and cooled distilled water until free of alkali. Columns were poured from a slurry of this resin in $CO_2$-free water. If not used at once, the column was rinsed again before use, since residual traces of alkali and of tertiary amine were leached from the resin on prolonged standing in the free-base form.

Zeo-Karb 226 (50 $\mu$ beads, hydrogen form; Permutit Co. Ltd.) was treated as described for the similar resin Amberlite XE-64 by Hirs, Moore & Stein (1953).

Zeo-Karb 226 (100-mesh beads, hydrogen form: Permutit Co. Ltd.) was graded by backwashing (Hamilton, 1958). Coarse and fine fractions, of about 10% of the total amount of resin each, were rejected, and the main fraction was then treated like the 50 $\mu$ bead resin. It was buffered by stirring with twice its volume of the appropriate buffer, and solid A.R. NaOH was then added to the stirred suspension of resin until the pH of the supernatant remained constant at the

original value. Columns of the resin were poured from a slurry made up in the same buffer.

*Preparation of the amine concentrate.* The solution or extract containing the amines, with 0·1% (v/v) of thiodiglycol (di-2-hydroxyethyl sulphide) added as an antoxidant, was passed through a column of De-Acidite FFX in the free-base form at a flow-rate of 20–40 ml./cm.²/min. (manufacturer's recommendation). This displaced the equilibrium between the chloride and hydroxide forms of the resin towards the chloride form by removal of hydroxyl ions, and was achieved, where necessary, by passing compressed $N_2$ into the reservoir above the column: the apparatus was built to withstand an excess of pressure of about 1 atm.

The effluent, followed by two equal rinses of $CO_2$-free water, was allowed to flow directly into a stirred suspension of Zeo-Karb 226 (50$\mu$ beads, hydrogen form) in water. A rough calculation indicated the amount of resin needed, allowing for 20% excess. When all the effluent and rinsings had been added, stirring was continued for an hour. The pH of the supernatant was then 6·5.

The Zeo-Karb 226 containing the amines was poured into a column, allowed to drain but not to dry out, and washed with two equal portions of water to remove neutral compounds. The combined organic and inorganic bases were eluted with 0·5 N-HCl, twice the volume at which the acid front emerged being used. The eluate was concentrated by vacuum-distillation at room temperature and evaporated to dryness *in vacuo* over a mixture of flake NaOH and granular $CaCl_2$, giving a concentrate containing amine hydrochlorides and ammonium and metal chlorides. Where the proportion of inorganic material was low, the concentrate was used as such, otherwise it was extracted with three successive portions of boiling 95% ethanol, leaving a residue of inorganic material. The ethanol extract was evaporated to dryness *in vacuo* before the next stage.

*Ion-exchange chromatography of the concentrate.* Zeo-Karb 226 (100-mesh beads, buffered at pH 7·3) was poured into a column to a depth of 110 cm. The column had a short side arm, closed with a self-sealing plug, near the top. When the top of the bed was level with the side arm the buffer (pH 7·3) was allowed to drain down to the top of the bed, and both the reservoir and the space above the bed were filled with buffer at pH 5·0. A solution of the amine concentrate in this buffer was injected through the plug with a syringe, and elution was allowed to proceed. Optimum conditions were as follows: load about 25 mg. of N/cm.² cross-sectional area, in not more than 2 ml. of buffer/cm.²; flow-rate 10–20 ml./cm.²/hr. At the end of the elution the whole column was at pH 5·0, and the pH of the effluent changed to this value from the initial pH 7·3. Elution volumes of some amines are shown in Table 1.

*Detection of amines in the effluent fractions.* Primary and secondary amines were determined by the modified Folin method of Blau & Robson (1957), by using 1 ml. of each fraction and adding 4 ml. of 50% ethanol, instead of taking 5 ml. of solution. The ethanol prevented precipitation of the coloured compounds formed from some of the higher-molecular-weight amines. Table 2 gives the colour yields and limits of linearity of some primary and secondary amines.

Table 1. *Elution volumes of some amines in column chromatography*

The amines were chromatographed as described in the text, some singly and others in mixtures. The elution volume is the total volume eluted at the amine peak with in each case a column of 0·8 cm. bore and depth 112·5 cm., filled with Zeo-Karb 226 buffered at pH 7·3, and eluted with buffer pH 5·0. Counting was started after rejection of the first 25 ml. after application of the sample.

| Amine | Elution volume (ml.) | Shape of peak | Recovery |
|---|---|---|---|
| Trimethylamine *N*-oxide | 12 | Very sharp | Quant. |
| Creatinine | 20 | Very sharp | Quant. |
| Tetramethylammonium | 79 | Sharp | Quant. |
| Diethylamine | 81 | Sharp | Quant. |
| *n*-Amylamine | 109 | Sharp | Quant. |
| *iso*Amylamine | 111 | Sharp | Quant. |
| Trimethylamine | 118 | Sharp | Quant. |
| Piperidine | 128 | Sharp | Quant. |
| *n*-Butylamine | 130 | Sharp | Quant. |
| Pyrrolidine | 148 | Sharp | Quant. |
| *n*-Propylamine | 150 | Sharp | Quant. |
| Dimethylamine | 162 | Sharp | Quant. |
| Ethylamine | 184 | Sharp | Quant. |
| Tyramine | 260 | Broad | 85% |
| Glucosamine | 270 | Broad | 85% |
| Methylamine | 280 | Broad | Quant. |
| Canavanine | 290 | Very broad | 80% |
| Ethanolamine | 300 | Broad | 80% |
| Adrenaline | 310 | Very broad | Not determined |
| Arginine | 330 | Broad | 70% |
| 3-Hydroxytyramine | 430 | Very broad | 90% |
| Noradrenaline | 460 | Very broad | 70% |
| 5-Hydroxytryptamine | 465 | Very broad | 80% |
| Ammonia | 490 | Broad | Quant. |
| pH 5·0 breakthrough | 600 | — | — |

Total N was determined by the micro-Kjeldahl method of Lang (1958); 1 ml. of each fraction was pipetted into a Pyrex tube, and the tubes were heated with the digestion mixture in a square aluminium block drilled with 100 holes.

*Desalting of amine solutions.* The fractions containing an amine peak were combined, made just acid to Congo red paper and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum volume of 0·025 N-HCl and the solution was injected through a self-sealing plug closing a short side-arm at the top of a column containing Zeo-Karb 226 (100-mesh beads, hydrogen form; column diameter 2·5 cm., bed depth 20 cm.; this column was suitable for desalting about 50 mg. of amine). The whole system including the reservoir was kept filled with 0·025 N-HCl, which was also used for the elution. Small portions of the fractions were withdrawn and analysed for total N as above, and elution was stopped after emergence of the amine, leaving the column ready for desalting once more. Fig. 1 shows an elution curve for a solution containing sodium citrate and piperidine. Where the volume of solution applied was large, or where the proportion of salt was high, it was sometimes necessary to evaporate the fractions containing amine down to small volume and repeat the desalting. The amine peak always emerged at the same volume for a given column. Evaporation of the combined fractions containing the amine left a residue of the crystalline amine hydrochloride.

Table 2. *Colour yields of some amines by the modified Folin method of Blau & Robson* (1957)

Extinction was measured in a Unicam SP. 500 spectrophotometer at a wavelength of 476 m$\mu$ in 10 mm. glass cells. The final solution contained 2 ml. of ethanol out of a total of 9 ml.

| Amine | Colour yield (extinction units/$\mu$mole) | Limiting amount ($\mu$moles) |
|---|---|---|
| Adrenaline | 0·15 | 5+ |
| *n*-Amylamine | 0·43 | 4 |
| *iso*Amylamine | 0·43 | 3 |
| Ammonia | 0·23 | 5+ |
| Arginine | 0·39 | 4 |
| *n*-Butylamine | 0·47 | 4 |
| 1:4-Diaminobutane | 0·84 | 2 |
| 1:5-Diaminopentane | 0·68 | 3 |
| Diethanolamine | 0·19 | 3 |
| Diethylamine | 0·19 | 4 |
| Dimethylamine | 0·53 | 4 |
| Ethanolamine | 0·40 | 4 |
| Ethylamine | 0·39 | 4 |
| Glucosamine | 0·12 | 5+ |
| Glycine | 0·49 | 5+ |
| Histamine | 0·36 | 3 |
| Hydroxyproline | 0·53 | 4 |
| 5-Hydroxytryptamine | 0·38 | 4 |
| 3-Hydroxytyramine | 0·38 | 3 |
| Methylamine | 0·42 | 4 |
| Noradrenaline | 0·38 | 4 |
| Phenethylamine | 0·48 | 2 |
| Piperidine | 0·56 | 4 |
| Piperidine-3-carboxylic acid (pipecolic acid) | 0·12 | 2 |
| Proline | 0·49 | 4 |
| *n*-Propylamine | 0·46 | 4 |
| Pyrrolidine | 0·53 | 3 |
| Sarcosine | 0·59 | 3 |
| Tryptamine | 0·35 | 6 |
| Tyramine | 0·43 | 3 |

*Paper chromatography.* Whatman no. 1 paper was developed by descending chromatography counter to the machine direction, after at least 2 hr. of equilibration in the vapour of the solvent. The following solvent systems were used: butan-1-ol–conc. HCl–water (7:2:1, by vol.; homogeneous; pH 1); butan-1-ol–acetic acid–water (4:1:5, by vol.; two phases; the mixture was refluxed for an hour and left to separate overnight; the lower layer was used for equilibration, and the upper, pH 3, for development); 2-methoxyethanol–propionic acid–water (14:3:3, by vol.; saturated with solid NaCl; homogeneous; pH 2; Herbst *et al.* 1958); butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol., homogeneous, pH 5; Waley & Watson, 1953); pyridine–pentan-1-ol–water (3:3:2, by vol.; homogeneous; pH 9). The developed chromatograms were dried in a current of air and sprayed with the following reagents: Folin's amino acid reagent, 1% 1:2-naphthoquinone-4-sulphonic acid (purified sodium salt) followed by saturated $KHCO_3$; ninhydrin–$Cu(NO_3)_2$ (Moffat & Lytle, 1959); alkaline ferricyanide–nitroprusside (Block, Durrum & Zweig, 1955) used without dilution; Ehrlich's reagent (Smith, 1953); diazotized *p*-anisidine (Sanger & Tuppy, 1951) modified by replacing half the *p*-anisidine with sulphanilamide; isatin (Boyarkin, 1956), developing the colours by heating at 100° for 10 min. after spraying; Sakaguchi reagent (Williams, 1951); Jaffé reagent, saturated picric acid followed by N-NaOH; Nessler's reagent (Koch & McMeekin, 1924). The papers were inspected under u.v. light, then hung in an atmosphere saturated with $I_2$ vapour, and finally sprayed after hanging in a current of air to remove $I_2$. The $I_2$ did not affect the colour reactions, except for easily oxidized amines. The $R_F$ values in Table 3 have been related to that of dimethylamine, which was arbitrarily chosen as convenient. This empirically improved their reproducibility, and involved measuring the $R_F$ of dimethylamine in each solvent from at least 30 measurements. 'Corrected solvent fronts' on subsequent chromatograms were drawn from the positions of reference spots of dimethylamine included at either end and in the centre of every sheet, and the $R_F$ values of all the other spots were

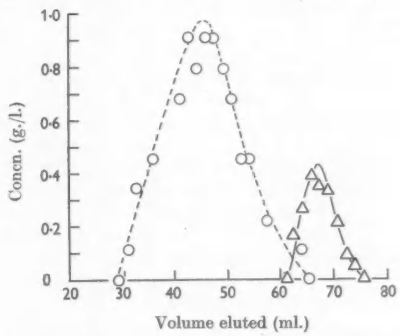


Fig. 1. Elution curve from a solution containing 100 mg. of trisodium citrate (dihydrate) and 5 mg. of piperidine hydrochloride in 3 ml. of water applied to a column (2·4 cm. × 18 cm.) of Zeo-Karb 226 (hydrogen form) and eluted with 0·025 N-HCl. Recoveries were quantitative within experimental error. ○, Sodium; △, piperidine.

Table 3. *Standardized $R_F$ values of amines*

$R_F$ values are related to those of dimethylamine (in bold type), as described in the text. The solvent systems are designated by letters as follows: A, butan-1-ol–acetic acid–water; B, butan-1-ol–acetic acid–water–pyridine; C, 2-methoxyethanol–propionic acid–water; D, pyridine–pentan-1-ol–water; E, butan-1-ol–conc. HCl–water. The spray reagents are abbreviated as follows: $I_2$, iodine vapour; NQS, Folin's reagent; NCN, ninhydrin–$Cu(NO_3)_2$; FCNP, alkaline ferricyanide–nitroprusside; EHR, Ehrlich's reagent; IS, isatin; UV, fluorescence under u.v. light; NN, diazotized *p*-anisidine–sulphanilamide; JAF, alkaline picric acid; NESS, Nessler's reagent; SAKA, Sakaguchi's reagent; IP, potassium iodoplatinate; for details of solvents and spray reagents see text. Numerical colour values refer to those on the 'Derwent' colour chart (see text and Reio, 1958).

Amines were applied to the paper as 0·5 % solutions of their salts (mainly the hydrochlorides, otherwise the bitartrates and acid citrates) in 50 % ethanol.

| Compound | 100 $R_F$ | | | | | Colours | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | $I_2$ | NQS | NCN | FCNP | EHR | IS | Other reagents |
| Acetylcholine | 39 | 45 | 78 | 43 | 46 | ++ | . | . | . | . | . | IP: 39 (24) |
| Adrenaline | 39 | 54 | 70 | 65 | 43 | ++ | 4 | . | 11 | . | 20 | NN: 9; UV: + |
| Agmatine | 06 | 35 | 42 | 04 | 24 | + | 68 | 26 | 22 | (2) | 26 | SAKA: 18 |
| 4-Amino-5-carbamoyl-glyoxaline | 40 | 55 | 64 | 50 | 34 | . | 25 | 70 | 70 (26) | 6 | 7 | NN: 65; IP: 9 |
| *p*-Aminobenzoic acid | 85 | 92 | 82 | 79 | 73 | + | 8 | 16 | . | 2 | . | UV: +; NN: 2 |
| 2-Amino-2-hydroxymethyl-propane-1:3-diol (tris) | 21 | 43 | 67 | 38 | 33 | . | (34) | . | . | . | . | . |
| 1-Aminopropan-2-ol | 34 | 48 | 69 | 33 | 43 | + | 31 | 24 | . | . | 19 | . |
| *N*-(3-Aminopropyl)-1:4-diaminobutane (spermidine) | 05 | 18 | 27 | 02 | 08 | ++ | 29 | 24 | . | . | 24 | IP: 71 |
| *p*-Aminosalicylic acid | 43 | 56 | 65 | 41 | 72 | ++ | 21 | 9 | 46 | 1 | 8 | NN: 57; UV: + |
| Ammonia* | 22 | 42 | 56 | 32 | 24 | . | 68 | . | 72 | (40) | . | NESS: 58 |
| *iso*Amylamine | 69 | 77 | 84 | 68 | 87 | + | 69 | 24 | . | (2) | 11 | IP: 72 |
| *n*-Amylamine | 68 | 83 | 87 | 71 | 92 | + | 27 | 26 | . | (1) | 12 | . |
| Aniline | 94 | 98† | 90 | 90† | 79 | + | 21 | . | . | 5 | . | . |
| Arginine | 14 | 26 | 38 | 12 | 20 | . | 70 | 26 | 22 | (1) | 19 | SAKA: 18 |
| Atropine | 69 | 81 | 92 | 68 | 86 | +++ | . | . | . | . | . | IP: 35 |
| Benzylamine | 63 | 76 | 84 | 68 | 74 | + | 33 | 24 | . | (1) | 22 | IP: 72 |
| Betaine | 27 | 32 | 59 | 18 | 44 | + | . | . | . | . | . | IP: (24) |
| Brucine | 61 | 70 | 77 | 60 | 64 | ++ | 7 | . | . | . | . | IP: 35 |
| *n*-Butylamine | 61 | 73 | 83 | 64 | 78 | ++ | 69 | 24 | . | (1) | 24 | . |
| Caffeine | 93 | 86 | 85 | 64 | 54 | ++ | . | . | . | . | . | UV: + |
| Canavanine | 14 | 19 | 36 | 07 | 16 | . | (52) | 26 | 22 | . | 24 | SAKA: 18 |
| *N*-(2-Chloroethyl)dibenzyl-amine (Dibenamine) | 86 | 90 | 78 | 93 | 96 | ++ | . | . | . | . | . | IP: (26) |
| Choline | 31 | 37 | 70 | 26 | 45 | ++ | . | . | . | . | . | IP: (71) |
| Citrulline | 16 | 26 | 48 | 11 | 28 | . | 69 | 26 | . | 1 | 24 | . |
| Creatine | 37 | 54 | 57 | 40 | 30 | + | . | . | 22 | . | . | . |
| Creatinine | 36 | 51 | 71 | 40 | 37 | . | (26) | . | 72 (14) | . | . | JAF: 9 |
| Cystamine‡ | 14 | 35 | 44 | 25 | 18 | +++ | 31 | 22 | . | (1) | 24 | IP: 1 |
| 1:4-Diaminobutane | 11 | 25 | 38 | 10 | 18 | ++ | 29 | 26 | . | (1) | 68 | IP: 72 |
| 1:2-Diaminoethane | 13 | 27 | 33 | 04 | 14 | + | 18 | 24 | . | (47) | 63 | IP: 72 |
| 1:5-Diaminopentane | 12 | 30 | 44 | 12 | 19 | ++ | 31 | 26 | . | (2) | 41 | IP: 72 |
| 1:3-Diaminopropane | 10 | 26 | 36 | 22 | 15 | + | 68 | 24 | . | . | 19 | IP: 72 |
| *NN'*-Di-(3-aminopropyl)-1:4-diaminobutane (spermine) | 02 | 02 | 12 | 43 | 05 | . | 26 | . | . | . | . | IP: 71 |
| Diethanolamine | 25 | 44 | 70 | 12 | 41 | + | 9 | . | . | . | . | UV: + |
| Diethylamine | 55 | 62 | 83 | 58 | 73 | ++ | 17 | . | . | . | 69 | . |
| 2-Dimethylaminoethanol | 32 | 44 | 71 | 43 | 45 | + | . | . | . | . | . | . |
| *N*-Dimethylaniline | 87 | 99† | 97 | .† | 84 | ++ | . | . | . | . | . | . |
| Dimethylamine (reference compound) | **33** | **43** | **70** | **30** | **47** | ++ | 11 | 26 | . | . | 12 | . |
| *NN*-Dimethylguanidine | 46 | 56 | 76 | 55§ 36 | 57 | ++ | . | . | 17 (24) | . | . | . |
| *NN*-Dimethylurea | 70 | 73 | 76 | 62 | 74 | + | . | . | . | (4)‖ | . | . |
| *NN'*-Dimethylurea | 62 | 66 | 74 | 61 | 83 | + | . | . | . | (4)‖ | . | . |
| Diphenylamine | 97 | 99 | 99 | 93 | 99 | +++ | 41 | . | . | 46 | 3 | UV: + |
| Ethanolamine | 26 | 41 | 65 | 23 | 35 | . | 33 | 26 | . | (2) | 14 | . |
| Ethylamine | 39 | 50 | 72 | 36 | 50 | ++ | 68 | 24 | . | . | 2 | . |
| Ethyleneimine | 42 | 56 | 74 | 10 | 52 | + | 26 | 24 | . | . | 9 | . |
| Glucosamine | 16 | 36 | 49 | 33 | 20 | . | 39 | . | . | (1) | 19 | UV: + |
| Glycocyamine | 30 | 28 | 56 | 16 | 42 | . | . | . | 14 (22) | . | . | . |
| Guanidine | 38 | 47 | 67 | 36 | 47 | ++ | . | . | 7 | . | . | UV: +; SAKA: 18 |
| Harmaline | 75 | 80 | 80 | 87 | 83 | ++ | 48 (51) | 4 | 51 (45) | 1 | 52 | UV: +; NN: 9; IP: 65 |
| Hexamethylenetetramine | 31 | 40 | 63 | 29 | 81 | ++ | . | . | . | . | . | IP: 18 |
| *cyclo*Hexylamine | 65 | 75 | 84 | 67 | 88 | ++ | 52 | . | . | . | 2 | . |
| Histamine | 13 | 33 | 34 | 31 | 18 | + | 70 | 24 | . | . | 24 | IP: 72; NN: 8 |
| Histidine | 12 | 24 | 25 | 14 | 13 | + | 69 | 24 | 18 | (1) | 24 | NN: 8 |
| 5-Hydroxytryptamine | 44 | 64 | 75 | 65 | 39 | ++ | 69 | 18 | 44 | 26 | 12 | NN: 12 |
| 3-Hydroxytyramine | 40 | 61 | 78 | 64 | 44 | +++ | 51 | 18 | 18 | (1) | 8 | NN: 8 |
| Indole | 96 | 99 | 92 | 94 | 99 | ++ | 21 | . | . | 23 | . | NN: 7 |
| Lysine | 10 | 20 | 37 | 07 | 17 | + | 26 | 26 | . | (1) | 35 | . |
| Methylamine | 30 | 40 | 65 | 31 | 38 | + | 68 | 26 | . | (2) | 13 | . |

Table 3 (*cont.*)

| Compound | 100 $R_F$ A | B | C | D | E | Colours $I_2$ | NQS | NCN | FCNP | EHR | IS | Other reagents |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2-Methylaminoethanol | 31 | 45 | 70 | 11 | 45 | + | 15 | . | . | . | 19 | . . |
| *N*-Methylaniline | 81 | 96 | 90 | 93 | 83 | ++ | 15 | . | . | . | . | . |
| Methylguanidine | 46 | 55 | 73 | 52§ 38 | 57 | + | . | . | 14 | . | . | SAKA: 18 |
| 2-Methylindole | 95 | 99 | 90 | 93 | 99 | ++ | 19 | . | . | 21 | . | UV: +; NN: 6 |
| 3-Methyindole (skatole) | 97 | 99 | 91 | 95 | 99 | ++ | 70 | . | . | 24 | . | UV: + |
| *N*-Methylpyridine | 37 | 39 | 72 | 26 | 44 | +++ | . | . | . | . | . | UV: +; IP: 38 |
| Methylurea | 61 | 64 | 74 | 58 | 73 | ++ | 18 | . | 57 | 2 | 57 | UV: + |
| α-Naphthylamine | 96 | 99 | 87 | 90 | 86 | ++ | 19 | . | . | 3 | . | UV: +; NN: 12 |
| β-Naphthylamine | 96 | 99 | 85 | 95 | 86 | ++ | 20 | . | . | 4 | . | UV: +; NN: 9 |
| Nicotine | 54 | 61 | 81 | 87 | 33 | ++ | . | . | . | . | . | IP: 39 |
| Noradrenaline | 30 | 50 | 66 | 59 | 31 | ++ | 69 | 5 | 11 | . | 20 | UV: +; NN: 10 |
| Ornithine | 09 | 19 | 30 | 07 | 13 | + | 31 | 26 | . | (1) | 35 | . . |
| Phenethylamine | 69 | 76 | 84 | 69 | 83 | + | 24 | 71 | . | (1) | 26 | IP: 71 |
| Piperidine | 53 | 61 | 77 | 53 | 65 | + | 14 | . | . | . | 47 | . |
| Piperidine-2-carboxylic acid (pipecolic acid) | 38 | 44 | 63 | 28 | 62 | + | 17 | . | . | . | 37 | . |
| *n*-Propylamine | 50 | 63 | 82 | 59 | 64 | + | 26 | 26 | . | (2) | . | . |
| Pyridine | 20 | 44† | 60 | 30† | 44 | . | . | . | . | . | . | IP: 38 |
| Pyridine-2-carboxylic acid (nicotinic acid) | 75 | 71 | 78 | 39 | 41 | + | . | . | . | . | . | . |
| Pyrrolidine | 43 | 53 | 74 | 47 | 58 | ++ | 13 | . | . | . | 41 | . . |
| Quinine | 77 | 91 | 97 | 80 | 74 | ++ | . | . | . | . | . | UV: +; IP: 28 |
| Quinoline | 90 | 43 | 86 | 92 | 69 | ++ | . | . | . | . | . | IP: 38 |
| Sparteine | 66 | 93 | 95 | 71 | 61 | ++ | . | . | . | . | . | IP: 31 |
| Trigonelline | 26 | 32 | 54 | 18 | 38 | +++ | . | . | . | . | . | IP: 34 |
| Tri-*n*-amylamine | 95 | 96† | 98 | 95† | 98 | ++ | . | . | . | . | . | IP: 71 |
| Trimethylamine | 30 | 67 | 65 | 55 | 50 | ++ | . | 69 | . | . | . | . |
| Trimethylamine *N*-oxide | 43 | 49 | 76 | 23 | 58 | ++ | 11 | . | . | . | 21 | . |
| Trimethylvinylammonium (neurine) | 37 | 41 | 77 | 26 | 47 | +++ | . | . | . | . | . | IP: 34 |
| Tri-*n*-propylamine | 83 | 89† | 98 | .† | 94 | ++ | . | . | . | . | . | IP: 72 |
| Tryptamine | 62 | 76 | 81 | 67 | 64 | +++ | 24 | 18 | . | 22 | 19 | UV: + |
| Tyramine | 55 | 72 | 80 | 67 | 62 | ++ | 68 | 69 | . | (1) | 70 | NN: 9 |
| Urea | 45 | 49 | 67 | 42 | 56 | . | (11) | . | 16 | 1 (48) | . | . |
| Yohimbine | 78 | 84 | 95 | 86 | 89 | +++ | 44 | . | . | . | . | UV: +; IP: 63 |

* Ammonia could only be detected in high concentrations.

† Pyridine, some tertiary and some aromatic amines are difficult to detect, except at high concentrations, after being run in solvents containing pyridine.

‡ Cysteamine gave the same colours and had the same $R_F$ values as cystamine, and it is assumed that it was rapidly oxidized to cystamine after application to the paper.

§ The methylated guanidines always gave two spots in this solvent.

‖ The dimethylureas were detectable only in high concentrations.

calculated from these fronts. The numbers in the table refer to those on the 'Derwent' colour chart (Reio, 1958) and Reio's conventions are used in Table 3. The colours could be described sufficiently well by comparing the spots directly with the chart, which was covered with a sheet of tracing paper, thus reducing the brilliance of its colours to about the same level as that of the spots on the paper.

*Paper electrophoresis.* Best results were obtained with Whatman no. 1 paper and 0·04 M-sodium barbiturate adjusted to pH 8·0. A potential gradient of 20 v/cm. was applied for an hour, and *N*-2:4-dinitrophenylethanolamine was used as an electroendosmosis marker. The amines were visualized after drying the paper strips in a current of air and spraying them with the reagents described above. The distances moved by some amines, corrected for electroendosmosis, are given in Table 4.

## RESULTS

Preliminary experiments with single compounds dissolved in 1% sodium chloride showed that passage through the strongly basic resin removed all the amino acids except arginine, which passed through in 95% yield. Other classes of compounds that were retained were urinary pigments, proteins, pyrimidines, purines and all anions. Urinary pigments were impossible to remove completely from the resin on regeneration, although this did not have an adverse effect on its subsequent performance.

In the second step the pH of the supernatant of the stirred suspension of Zeo-Karb 226 stayed near neutrality and did not fall below 6·5 even at the end of the experiment. When chloride appeared in the effluent from the first column, owing to too low a flow-rate, the pH of the supernatant fell below 6·5, with a consequent possibility that some of the weaker bases were not adsorbed.

The recovery of the amines in all the stages up to and including the acid elution was found, in trial experiments, to be quantitative within experimental error, provided that the washing at each stage was adequate. Some losses occurred during the chromatographic separation for amines eluted

after methylamine (see Table 1). Also, amines with more than one basic group were not eluted from the resin under these conditions. Thus histamine, 1:4-diaminobutane and 1:5-diaminopentane gave no peaks before the pH 5 breakthrough, and satisfactory conditions for their elution have not yet been found.

Table 4. *Relative mobilities of some amines*

Electrophoresis was carried out on Whatman no. 1 paper strips with 0·04M-sodium barbiturate buffer, adjusted to pH 8·0, at 20v/cm. The distances are those moved by the amines in 1 hr., corrected for electroendosmosis.

| Amine | Distance moved (cm.) |
|---|---|
| Adrenaline | 3·5 |
| *n*-Amylamine | 7·0 |
| *iso*Amylamine | 6·8 |
| Arginine | 5·1 |
| *n*-Butylamine | 7·2 |
| Creatinine | 0·7 |
| 1:4-Diaminobutane | 10·2 |
| 1:5-Diaminopentane | 10·4 |
| Diethanolamine | 6·1 |
| Diethylamine | 7·9 |
| Dimethylamine | 11·7 |
| Ethanolamine | 8·5 |
| Ethylamine | 11·4 |
| Glucosamine | 2·5 |
| Histamine | 6·2 |
| Hydroxylamine | 1·4 |
| 5-Hydroxytryptamine | 3·1 |
| 3-Hydroxytyramine | 4·3 |
| Methylamine | 13·4 |
| Nicotine | 3·6 |
| Noradrenaline | 3·5 |
| Phenethylamine | 5·9 |
| Piperidine | 6·6 |
| *n*-Propylamine | 8·1 |
| Pyrrolidine | 9·4 |
| Tryptamine | 4·7 |
| Tyramine | 5·2 |

A useful feature of the method for desalting amines is that it worked not only with aliphatic and aromatic amines, but also with glutamic acid and arginine, which suggests that it is widely applicable to compounds containing amino groups.

The scope of these methods is indicated by Fig. 2, which shows elution curves (primary and secondary amines only) obtained from amine concentrates of 24 hr. urine samples. The first of these was obtained

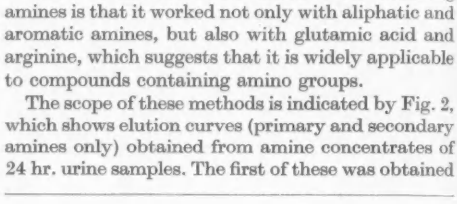


Fig. 2. Elution curves of the primary and secondary amines in concentrates corresponding to 500 ml. of urine applied to columns (2·0 cm. × 100 cm.) of Zeo-Karb 226 buffered at pH 7·3 and eluted with buffer at pH 5·0. (*a*), Curve obtained from urine (24 hr. volume, 1800 ml.) from a normal male on a normal diet; (*b*), curve from urine (24 hr. volume, 900 ml.) obtained from a normal male after a 5-day fast. *A*, Piperidine; *B*, dimethylamine; *C*, methylamine.

Table 5. *Excretion of piperidine, dimethylamine and methylamine*

The figures for each amine, as the free base, were calculated from elution curves obtained by the methods described in the text. The values for the normal diet are the average values from four different 24-hr. samples, with the range given underneath in parentheses. The values after fasting are from only one experiment.

| Amine | Source | Excretion (mg./ml.) | Excretion (mg./24 hr.) | Reference |
|---|---|---|---|---|
| Piperidine | Normal human | 3–20 | . | Euler (1945) |
| | Normal male (normal diet) | 3·8 (2·7–5·1) | 5·7 (4·7–7·1) | Present work |
| | Normal male (fasting) | 0 | 0 | Present work |
| Dimethylamine | Normal human | 5–40 | . | Löffler (1935) |
| | Normal human | 5 | . | Dowden (1938) |
| | Normal male (normal diet) | 10·9 (9·7–11·7) | 17·0 (15·2–19·3) | Present work |
| | Normal male (fasting) | 14·0 | 12·6 | Present work |
| Methylamine | Normal male | 82 | . | Kapeller-Adler & Krael (1931) |
| | Normal female | 100–150 | . | Kapeller-Adler & Toda (1932) |
| | Normal male (normal diet) | 3·3 (2·7–3·6) | 5·0 (4·6–6·0) | Present work |
| | Normal male (fasting) | 4·4 | 4·0 | Present work |

with the subject on a normal diet, the second on the fifth day of total fasting. The three major peaks were identified by the methods described as piperidine, dimethylamine and methylamine, all well established urinary constituents. Table 5 compares the excretion of these three amines with values from the literature.

The figures were calculated by measuring the areas under the peaks of the elution curve and correcting them for the total volumes of the 24 hr. specimens. Table 1 shows that glucosamine, methylamine and ethanolamine are not well resolved. It is therefore possible that the figures for the areas of the methylamine peaks may include small contributions from the other two amines, which occur in urine (Luck & Wilcox, 1953; Boas, 1956).

The most significant feature of these results is the absence of piperidine after fasting, which indicates that it is of dietary origin. It appears to be related structurally to lysine, from which it could arise by two alternative routes. The first is via the decarboxylation product, cadaverine, which was found, when fed to rabbits, to increase piperidine excretion, unlike lysine itself (Nordenström, 1951). The other pathway from lysine is that which Rothstein and his group (Rothstein & Miller, 1954; Rothstein & Greenberg, 1959) traced in the rat, via α-aminoadipic acid to glutaric acid. Pipecolic acid is an intermediate on this route, and on decarboxylation would also give piperidine.

Little is known about the origin of urinary amines, although they are generally regarded as arising from amino acids by decarboxylation. There is, however, a suggestion that methylamine and dimethylamine might be breakdown products of creatinine (Kapeller-Adler & Toda, 1932). The values of their excretion after fasting are not much lower than the normal ones, so that these two amines certainly appear to be derived from endogenous sources.

## DISCUSSION

The methods developed in the present work were designed with the aim of being general for amines, and of minimizing the formation of alteration products. For this reason extreme conditions of temperature and of pH were avoided as much as possible. These considerations also governed the approach to the resolution of the amine concentrate. The chromatography of the dinitrophenyl derivatives (Asatoor & Dalgliesh, 1959) is limited to primary and secondary amines, and gas–liquid partition chromatography (James, Martin & Smith, 1952; James, 1952), although being potentially more rapid and convenient, is restricted to volatile amines. Weber (1951) used paper electrophoresis, but on the whole his results were disappointing. The use of ion-exchange chromatography provided a more general method, enabled the amines to be isolated as their hydrochlorides, and was capable of application on any scale.

## SUMMARY

1. A method has been developed for treating biological fluids and extracts with ion-exchange resins to prepare a fraction consisting predominantly of the amines.

2. The resolution of this fraction has been obtained by the use of buffered columns of a weakly acidic ion-exchange resin.

3. Amines separated in this way have been isolated as their hydrochlorides by a chromatographic desalting technique, and identified by paper chromatography and paper electrophoresis.

4. The scope of these methods is illustrated with reference to the analysis of amines in urine, and figures are presented for the excretion of piperidine, dimethylamine and methylamine, both normally and after fasting.

I should like to thank Professor W. Robson for his encouragement and continued interest in this work.

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# The Recovery of Injected Antigens from Rat Spleens

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The fate of heterologous proteins in experimental animals has been widely studied, particularly since the use of radioactive isotopes became widespread. The subject has been comprehensively reviewed by Haurowitz (1953, 1960) and Coons (1954). Protein-bound radioactivity has been shown to persist in the organs of rabbits injected with trace-labelled native proteins for up to 93 days (Crampton, Reller & Haurowitz, 1953). A similar persistence of radioactivity after injection of an extensively modified protein (anthranilylazo-ovalbumin) has also been demonstrated (Crampton, Reller & Haurowitz, 1952), despite the well-known differences between the immediate fate of native and extensively modified proteins in rabbits (Dixon, Bukantz & Dammin, 1951). Extensively modified azoproteins and iodoproteins leave the blood stream very rapidly (Haurowitz & Crampton, 1952; Francis & Hawkins, 1957), and during the first week after injection are found in much higher concentration in the tissues than are native proteins.

When the tissues in which antigen had been deposited were fractionated by differential centrifuging, Haurowitz & Crampton (1952) and Crampton *et al.* (1953) found that most of the antigen was associated with the mitochondrial fraction except during the first few minutes after injection. However, few studies have been reported of the chemical nature of the radioactivity retained in the tissues, so that little is definitely known about the persistence of the antigen itself. McMaster & Kruse (1951) have shown by a very sensitive immunological technique that unlabelled bovine $\gamma$-globulin injected into mice can be recovered from their livers in an immunologically reactive form 101 days later. Haurowitz & Walter (1955) injected [$^{35}$S]-sulphanilylazo bovine $\gamma$-globulin into rabbits, obtained protein from the liver 4 weeks later and hydrolysed it. Most of the $^{35}$S was probably in the form of sulphanilylazotyrosine. This suggested that the determinant group of the protein was still intact. When Garvey & Campbell (1957) injected rabbits with [$^{35}$S]sulphanilylazo bovine serum albumin they recovered from the livers, up to 130 days later, labelled material which was highly active in sensitizing guinea pigs to the injected antigen. This material was associated with substances having an absorption maximum in the ultraviolet at 257·5 m$\mu$. It is difficult to decide whether this material is likely to play a part in the development of the immune response, as the liver is probably not the site of antibody synthesis.

The work reported here was undertaken to try to provide more information about the chemical nature of the protein-bound radioactivity persisting in the spleen (since this organ is probably a site of antibody synthesis) after the injection of radioactive proteins. Two native heterologous proteins, trace-labelled with diazotized [$^{35}$S]sulphanilic acid, and one extensively modified protein, have been injected into rats. For comparison, trace-labelled rat serum albumin has also been injected. Determination of the amount of protein-bound $^{35}$S precipitable from extracts made from the spleens of these rats by the appropriate rabbit antibody at various times after injection provided a measure of the persistence of free antigen. In view of Garvey & Campbell's findings, and also because of the well-known association of nucleic acids with protein synthesis, extraction and fractionation of the spleens were designed in such a way that extracts

containing ribonucleoproteins and deoxyribonucleoproteins (Chargaff, 1955; Kay & Dounce, 1953) were obtained.

A preliminary account of some of this work has been published (Hawkins & Haurowitz, 1959).

## MATERIALS AND METHODS

*Sulphanilic acid.* This was prepared from aniline bisulphate and $H_2{}^{35}SO_4$ (20–50 mc, carrier-free, obtained from the U.S. Atomic Energy Commission, or The Radiochemical Centre, Amersham, Bucks.) by the method of Ingraham (1951).

*Proteins.* Bovine γ-globulin (Pentex Inc.), crystalline bovine serum albumin (Pentex Inc. or Armour and Co. Ltd.) and rat serum albumin [the fraction precipitating between half and full saturation of rat serum with $(NH_4)_2SO_4$, and shown by paper electrophoresis to contain about 90% of albumin and 10% of globulins] were trace-labelled with diazotized [$^{35}$S]sulphanilic acid as described by Crampton *et al.* (1953).

Anthranilylazo bovine serum albumin was made by coupling diazotized anthranilic acid (25 mg.) to bovine serum albumin (100 mg.) at pH 9 for 3 hr. at 0°, these amounts having been shown to produce maximal coupling of the azo compound in trial experiments. As [$^{35}$S]-sulphanilic acid was available, it was used to label anthranilylazo bovine serum albumin by allowing bovine serum albumin (50 mg.) to react first with diazotized [$^{35}$S]sulphanilic acid (2·5 mg.) and then with diazotized non-radioactive anthranilic acid (12 mg.). The product was freed from radioactive non-protein substances by three precipitations at pH 4·5, followed by dialysis against 0·15 M-NaCl.

*Antisera.* Antisera were obtained from rabbits given two or more intramuscular injections of 5 ml. of 2% alum-precipitated proteins (Proom, 1943). The maximum amount of labelled antigen that could be precipitated completely by an antiserum was determined by measuring the radioactivity in precipitates obtained after incubating increasing amounts of antigen with samples of the antiserum for 1 hr. at 37°, followed by keeping for 5–7 days at 4°.

*Injection of rats and treatment of spleens.* In some early experiments rats were anaesthetized with ether and injections were made into the femoral vein, but in later experiments the injections were intraperitoneal. Each rat was injected with 3·0 mg. of protein. At suitable times after injection, rats were killed with coal gas. Blood (2–3 ml.) was collected in an oxalated syringe by cardiac puncture and the plasma was separated by centrifuging. The spleen was rapidly removed, weighed and cooled on ice. Each spleen was finely chopped with scissors and homogenized with five times its weight of 0·10 M-NaCl–0·05 M-sodium citrate (saline–citrate; Chargaff, 1955) in a cooled Potter-type homogenizer with a Teflon pestle. The extractant contained about 150 μg. of the same protein (unlabelled) as that with which the animal had been injected. The homogenate was centrifuged at 10 000 *g* at 0° for 20 min. and the supernatant sucked off. The residue was re-extracted with the saline–citrate (three times the weight of the spleen), centrifuged as before and the extracts were combined. In some cases the residue was further extracted twice with water which had been neutralized to pH 7·0 (three times the weight of the spleen; Chargaff, 1955). A final extraction with this water (five times the weight of the spleen) was allowed to proceed overnight at 4° before centrifuging and removal of the highly viscous supernatant. In some cases the deoxyribonucleoprotein in this extract was purified by adding 4 M-NaCl to a final concentration of 0·15 M-NaCl, centrifuging and redissolving in water or M-NaCl.

Two samples of each extract were pipetted into tared 50 ml. centrifuge tubes. To one was added antiserum (generally 1–2 ml.) to the protein with which the rat had been injected. The amount of antiserum was chosen so that it would precipitate slightly more of the carrier protein than was added during the homogenization. To the second tube was added 100 μg. of an unrelated antigen (usually ovalbumin), followed by 1 ml. of an antiserum to this antigen. The tubes were incubated for 1 hr. at 37°, kept for 5–7 days at 4° and centrifuged, and the precipitate washed with 2 × 1·0 ml. of 0·15 M-NaCl and dissolved in about 1 ml. of 0·02 N-NaOH.

*Determination of radioactivity.* When rats had been injected with bovine γ-globulin, the protein in the spleen fractions and precipitins was precipitated with trichloroacetic acid at a final concentration of 8% (w/v) and washed successively with 8% trichloroacetic acid (twice), acetone (twice) and ether. The product was dried for 1 hr. at 105°. This method does not work for albumin since the trichloroacetate of this protein is readily soluble in acetone (Levine, 1954). Precipitation was therefore effected by heating the protein in a boiling-water bath for 5 min., then adding 1 vol. of methanol–0·2 M-acetate buffer, pH 5·2 (6:1, v/v), and leaving overnight at 4°. The precipitated protein was then centrifuged and washed with methanol–0·02 M-acetate buffer, pH 5·2 (10:1, v/v), methanol, acetone and ether and dried by heating as described above. Trial experiments showed that this method gave a satisfactory recovery of serum albumin. The method was also used in experiments with anthranilylazo bovine serum albumin. The protein powders were weighed, finely ground and 30 ± 0·2 mg. samples were weighed on nickel-plated planchets for counting in a windowless Geiger counter.

The radioactivity injected varied between $5 \times 10^5$ and $3 \times 10^6$ counts/min. in different experiments. The actual counts/min. measured on protein powders from different fractions varied widely, and the accuracy of measurements of figures recorded in the Tables as less than 50 counts/min. was low.

When extracts of spleen fractions were made with $HClO_4$, portions (3·0 ml.) of the extracts were pipetted on to 1·0 g. of finely ground *p*-terphenyl (L. Light and Co. Ltd.) in a glass dish and counted with a scintillation counter (Steinberg, 1959). A drop of the neutral detergent Stergene was added to enable the $HClO_4$ extract to wet the hydrophobic *p*-terphenyl.

*Calculation and expression of results.* The protein-bound $^{35}$S in a sample of the plasma of each rat was determined by the method outlined above. For plotting the graphs in Figs. 1 and 2, a correction to the observed counts/min./ml. of plasma was made, since the rats were not all of the same weight when they were killed and would therefore have had different plasma volumes. In each case the counts/min./ml. of plasma were multiplied by 0·0415 × the body wt. (g.), since the plasma volume of the rat is given as 41·5 ml./kg. body wt. (Spector, 1956).

In discussing the amount of $^{35}$S in each spleen it is helpful to have some figure that relates the amount of $^{35}$S to both

the weight of the spleen and the $^{35}S$ content of the plasma. For this purpose the term 'protein-distribution factor' has been coined. This is equal to (total counts/min./g. of spleen)/(counts/min./ml. of plasma), and is thus a similar term to the 'apparent blood volume' used by Francis, Hawkins & Wormall (1957). If all the antigen in the spleen is in the extracellular fluid the protein-distribution factor would equal the volume of the extracellular fluid of the spleen in ml./g. The actual volume of the extracellular fluid of the spleen is not known, though our best estimate is 0·28 ml./g. (see Discussion and Table 8). Thus it seems reasonable to conclude that values of 0·5 or greater for the protein-distribution factor indicate that some of the antigen in the organ must have left the extracellular-fluid compartment. There are a number of ways in which this could occur, e.g. penetration into or adsorption on to the cells, precipitation of antigen–antibody complexes if the animal is immune (cf. Garvey & Campbell, 1954) or incorporation of $^{35}S$-containing peptides into tissue proteins. At present, we have no means of distinguishing between these possibilities.

## RESULTS

*Bovine γ-globulin as antigen.* The logarithm of protein-bound $^{35}S$ activity in the plasma of the rats injected with bovine γ-globulin is plotted against the time after injection in Fig. 1. Most of the points lie on, or reasonably close to, a straight line, from which it can be calculated that the half-life of bovine γ-globulin in the plasma of the rat is 2·5 days. The points from the rats killed 8 and 10 days after injection are well below the line, and this suggests that there may have been immune elimination of some of the antigen from the plasma of these animals (Talmage, Dixon, Bukantz & Dammin, 1951), even though there was no evidence of immune elimination of the antigen from the plasma of animals killed at later times.

Table 1 presents the protein-distribution factors of the spleens of these rats. These figures suggest that some of the antigen has left the extracellular fluid in rats killed 8, 10 and 18 days after injection.

Table 2 shows the data obtained on fractionating the spleens of these rats and on precipitating bovine γ-globulin in the saline–citrate extracts with

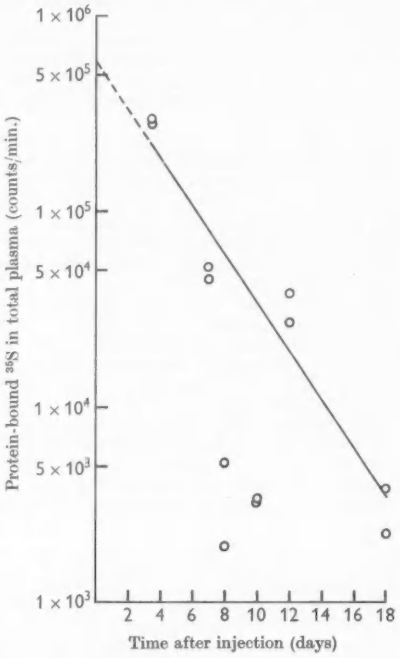
1 × 10⁶
5 × 10⁵
1 × 10⁵
5 × 10⁴
1 × 10⁴
5 × 10³
1 × 10³
Protein-bound ³⁵S in total plasma (counts/min.)
2 4 6 8 10 12 14 16 18
Time after injection (days)

Fig. 1. Persistence of bovine γ-globulin, trace-labelled with $^{35}S$, in rat plasma after intravenous injection.

Table 1. *Protein-bound $^{35}S$ in spleens and plasma samples of rats injected intravenously with $^{35}S$-labelled bovine γ-globulin*

All counts/min. are corrected to $10^6$ counts/min. injected.

| Time after injection (days) | Counts/min. in spleen | Wt. of spleen (g.) | Counts/min./ml. of plasma | Protein-distribution factor* |
|---|---|---|---|---|
| 3·5 | 563 | 0·42 | 4810 | 0·27 |
| 3·5 | 723 | 0·49 | 5310 | 0·28 |
| 7 | 565 | 0·94 | 1140 | 0·53 |
| 7 | 744 | 1·06 | 945 | 0·74 |
| 8 | 174 | 1·82 | 107 | 0·87 |
| 8 | 174 | 2·23 | 35 | 2·25 |
| 10 | 188 | 2·20 | 82 | 1·04 |
| 10 | 202 | 1·71 | 73 | 1·62 |
| 12 | 326 | 0·95 | 769 | 0·45 |
| 12 | 255 | 1·57 | 543 | 0·30 |
| 18 | 236 | 0·60 | 61 | 6·46 |
| 18 | 391 | 1·32 | 105 | 2·82 |

* (Total counts/min./g. of spleen)/(counts/min./ml. of plasma) (see Materials and Methods section).

rabbit anti-bovine γ-globulin. The small amount of radioactivity (usually about 15% of the total) extracted by the two short extractions with water is not shown. In all cases about half the total protein-bound $^{35}S$ in the spleen was extractable with saline–citrate. However, it was only during the first 7 days after injection that a substantial proportion of this was precipitable with anti-bovine γ-globulin. Thereafter very little was precipitable. Non-specific precipitation of $^{35}S$ in the presence of an ovalbumin–anti-ovalbumin precipitate was so small as to be negligible in all cases. The overnight aqueous extraction yielded a viscous liquid which is presumed to be mainly deoxyribonucleoprotein (a fraction obtained in the same way from another rat injected with bovine serum albumin had a nucleic acid-type spectrum with a maximum at 258 mμ and minimum at 235 mμ). Inconsistent results were obtained when this fraction was treated with anti-bovine γ-globulin, for although radioactivity was frequently precipitated, equal or greater amounts of radioactivity were also precipitated after the addition of the unrelated ovalbumin–anti-ovalbumin system. When the deoxyribonucleoprotein was purified by precipitation with 0·15 M-NaCl, followed by solution again in M-NaCl two or three times, most of the radioactivity passed into the 0·15 M-NaCl extract.

After removal of the bovine γ-globulin–anti-bovine γ-globulin precipitates from the saline–citrate extracts, samples of some of them were brought to pH 4·5 by the addition of ice-cold N-HCl (Kay & Dounce, 1953) and the precipitate was centrifuged, washed with 0·10 M-NaCl–0·05 M-sodium citrate adjusted to pH 4·5, and the protein-bound radioactivity in the precipitate determined. Table 3 shows that appreciable amounts of radioactivity were precipitated by this procedure, and that the percentage of radioactivity precipitated in this way was greater in the spleens of rats killed at longer times after injection.

*Bovine serum albumin as antigen.* When the logarithm of activity of protein-bound $^{35}S$ in the plasma is plotted against the time elapsed since the

Table 2. *Protein-bound $^{35}S$ in fractions from spleens of rats injected intravenously with $^{35}S$-labelled bovine γ-globulin*

All figures are counts/min. corrected to $10^6$ counts/min. injected. Anti-Bγ, anti-bovine γ-globulin; OA, ovalbumin.

| Time after injection (days) | Counts/min. extracted by saline–citrate | Counts/min. precipitated from saline–citrate extract in the presence of | | Counts/min. in aqueous extract | Counts/min. in residue |
|---|---|---|---|---|---|
| | | Anti-Bγ | OA–anti-OA | | |
| 3·5 | 367 | 219 | 7 | 51 | 83 |
| 3·5 | 446 | 309 | 5 | 85 | 103 |
| 7 | 260 | 186 | 5 | 51 | 145 |
| 7 | 326 | 237 | 6 | 67 | 230 |
| 8 | 111 | 10 | — | 22 | 23 |
| 8 | 96 | 7 | 0 | 20 | 36 |
| 10 | 120 | 11 | 3 | 16 | 35 |
| 10 | 139 | 8 | 4 | 19 | 30 |
| 12 | 145 | 11 | 2 | 44 | 80 |
| 12 | 108 | 9 | 2 | 21 | 65 |
| 18 | 83 | 8 | 1 | 26 | 92 |
| 18 | 216 | 2 | 1 | 51 | 60 |

Table 3. *Protein-bound $^{35}S$ in saline–citrate extracts of rat spleens after intravenous injection of $^{35}S$-labelled bovine γ-globulin*

All counts/min. are corrected to $10^6$ counts/min. injected. The extracts had previously been freed of $^{35}S$-labelled bovine γ-globulin by removal of the precipitate formed on addition of anti-bovine γ-globulin.

| Time after injection (days) | Counts/min. in saline–citrate extract after treatment with anti-bovine γ-globulin | Counts/min. precipitated at pH 4·5 | Percentage of $^{35}S$ of extract precipitated at pH 4·5 |
|---|---|---|---|
| 3·5 | 148 | 44 | 30 |
| 3·5 | 137 | 30 | 22 |
| 8 | 101 | 54 | 53 |
| 8 | 89 | 63 | 71 |
| 10 | 109 | 60 | 61 |
| 10 | 131 | 62 | 51 |

injection, the curve in Fig. 2 is obtained. The route of injection does not seem to make any significant difference in the plasma content of protein-bound $^{35}S$, and the main portion of the curve is a straight line with a half-life of 2·0 days. The points for one of the rats killed after 3 days and one after 9 days suggest the possibility of immune elimination, since they are below the line.

Table 4 shows the protein-distribution factors of the spleens of these rats. All that were killed 3 or more days after the injection clearly show evidence of considerable passage of antigen out of the extracellular fluid in spite of there being no evidence of immune elimination of the antigen from the plasma except in two cases.

When the spleens were fractionated and the free antigen in the saline–citrate extracts was determined, the results shown in Table 5 were observed. In all but three cases more than half of the total $^{35}S$ present in the spleen appeared in the saline–citrate extract, but there seems to be a tendency for the percentage extracted to fall as the time after injection increases. One day after injection just over half the $^{35}S$ in the saline–citrate extract was specifically precipitable by anti-bovine serum albumin, but this proportion fell rapidly until at 4 days it was only 5–10 %. Thereafter only very small amounts were precipitable and these were probably not significant, for in many cases there was measurable non-specific precipitation in the presence of the unrelated ovalbumin–anti-ovalbumin system. No activity was precipitated by anti-bovine serum albumin from the aqueous extracts on the occasions when they were tested.

Adjusting the pH of the saline–citrate extracts to 4·5 after removal of the free bovine serum albumin resulted in the precipitation of considerable amounts of protein-bound $^{35}S$ (Table 6). A higher percentage of the $^{35}S$ was precipitated from extracts in which there had been very little free bovine serum albumin precipitable by anti-bovine serum albumin.

Trichloroacetic acid (to a final concentration of 8 %) was added to samples of the residues of some spleens, and the precipitated protein was centrifuged. This precipitate was then washed twice with acetone to remove proteins which form acetone-

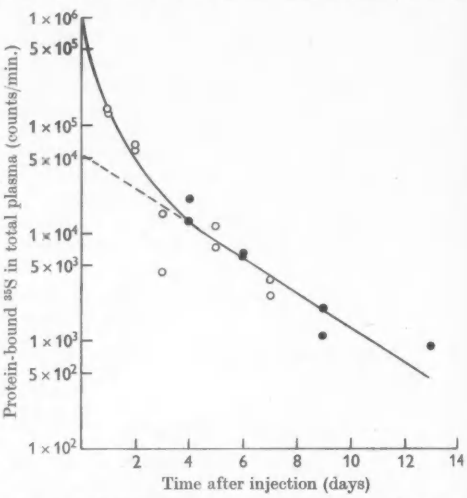


Fig. 2. Persistence of bovine serum albumin, trace-labelled with $^{35}S$ in rat plasma: ○, after intraperitoneal injection; ●, after intravenous injection.

Table 4. *Protein-bound $^{35}S$ in spleens and plasma samples of rats injected with $^{35}S$-labelled bovine serum albumin*

All counts/min. are corrected to $10^6$ counts/min. injected.

| Time after injection (days) | Counts/min. in spleen | Wt. of spleen (g.) | Counts/min./ml. of plasma | Protein-distribution factor* |
|---|---|---|---|---|
| 1 | 2051 | 0·79 | 14 910 | 0·17 |
| 1 | 1612 | 0·73 | 14 950 | 0·15 |
| 2 | 1256 | 1·03 | 6 720 | 0·18 |
| 2 | 2060 | 0·82 | 6 650 | 0·38 |
| 3 | 1660 | 0·71 | 1 905 | 1·23 |
| 3 | 1635 | 0·78 | 652 | 3·22 |
| 4 | 1061 | 0·68 | 1 675 | 0·93 |
| 4 | 1789 | 1·00 | 1 165 | 1·53 |
| 5 | 1443 | 0·87 | 1 640 | 1·01 |
| 5 | 1110 | 0·80 | 1 080 | 1·28 |
| 6 | 691 | 0·94 | 603 | 1·22 |
| 6 | 833 | 1·04 | 596 | 1·34 |
| 7 | 887 | 0·66 | 509 | 2·64 |
| 7 | 1001 | 1·25 | 409 | 1·96 |
| 9 | 1209 | 1·97 | 98 | 6·27 |
| 9 | 1177 | 1·13 | 189 | 5·51 |
| 13 | 1222 | 3·20 | 80 | 4·79 |

* See footnote to Table 1.

soluble trichloroacetates, and the residue was then treated for protein-bound $^{35}S$ in the usual way. Table 6 shows that very little $^{35}S$ was removed from the residues by this treatment. The apparent increase in $^{35}S$ in the residue of one spleen after this treatment is attributed to experimental error.

*Anthranilylazo bovine serum albumin as antigen.* This antigen disappeared very rapidly from the plasma of the injected rats (Table 7). No attempt has been made to calculate the protein-distribution factors of the spleens of these animals, but they would be very high. Table 7 also shows that less than half of the protein-bound $^{35}S$ was extractable from the spleen at all times after injection. Furthermore, no significant amounts of antigen were in a free state, precipitable by anti-anthranilylazo bovine serum albumin. In some cases there is a considerable discrepancy between the amounts of $^{35}S$ found in the saline–citrate extracts after treatment with anti-anthranilylazo bovine serum albumin and after addition of ovalbumin–anti-ovalbumin.

Because no antigen was precipitated by anti-anthranilylazo bovine serum albumin even at a short time after injection, a control experiment was performed in which labelled anthranilylazo bovine serum albumin (11·9 $\mu$g.) was added to homogenates made with saline–citrate from the spleens of two normal rats. This amount of antigen was calculated to be of the same order of magnitude as that found in the spleen 2 days after injection of 3 mg. of antigen. The homogenates were centrifuged and the residues extracted again in the normal way and precipitin tests were set up on the saline–citrate

Table 5. *Protein-bound $^{35}S$ in fractions from spleens of rats injected with $^{35}S$-labelled bovine serum albumin*

All figures are counts/min. corrected to $10^6$ counts/min. injected. BSA, Bovine serum albumin; OA, ovalbumin.

| Time after injection (days) | Counts/min. extracted by saline–citrate | Counts/min. precipitated from saline–citrate extract in the presence of | | Counts/min. in deoxyribo-nucleoprotein | Counts/min. in residue |
|---|---|---|---|---|---|
| | | Anti-BSA | OA–anti-OA | | |
| 1 | 1622 | 840 | 0 | — | 429 |
| 1 | 1300 | 716 | 0 | — | 312 |
| 2 | 881 | 366 | 0 | — | 375 |
| 2 | 1382 | 655 | 14 | — | 678 |
| 3 | 1067 | 210 | 50 | — | 593 |
| 3 | 1192 | 265 | 43 | — | 443 |
| 4 | 604 | 70 | 17 | 102 | 259 |
| 4 | 1020 | 56 | 10 | 162 | 291 |
| 5 | 937 | 49 | 40 | — | 506 |
| 5 | 715 | 39 | 30 | — | 395 |
| 6 | 385 | 29 | 4 | 61 | 140 |
| 6 | 408 | 28 | 4 | 76 | 209 |
| 7 | 578 | 54 | 33 | — | 304 |
| 7 | 400 | 33 | 17 | — | 601 |
| 9 | 654 | 16 | 17 | 66 | 249 |
| 9 | 641 | 28 | 27 | 64 | 280 |
| 13 | 522 | 17 | 8 | 118 | 300 |

Table 6. *Protein-bound $^{35}S$ in fractions of rat spleens after injection of $^{35}S$-labelled bovine serum albumin*

All counts/min. are corrected to $10^6$ counts/min. injected. BSA, Bovine serum albumin.

| Time after injection (days) | Counts/min. in saline–citrate extract after treatment with anti-BSA* | Counts/min. precipitated from saline–citrate extract at pH 4·5 | Percentage of $^{35}S$ of saline–citrate extract precipitated at pH 4·5 | Total counts/min. in residue | Counts/min. in residue after treatment with trichloroacetic acid and acetone |
|---|---|---|---|---|---|
| 1 | 782 | 215 | 27 | — | — |
| 1 | 584 | 134 | 23 | — | — |
| 2 | 515 | 153 | 30 | — | — |
| 4 | 534 | 219 | 41 | 259 | 171 |
| 4 | 964 | 491 | 51 | 291 | 228 |
| 6 | 356 | 160 | 45 | 140 | 129 |
| 6 | 380 | 144 | 38 | 209 | 177 |
| 9 | 638 | 312 | 49 | 249 | 206 |
| 9 | 613 | 300 | 49 | 280 | 317 |
| 13 | 505 | 333 | 66 | 300 | 224 |

* The saline–citrate extracts had previously been freed of $^{35}S$-labelled bovine serum albumin by removal of the precipitate formed on addition of anti-bovine serum albumin.

extracts. Table 7 (see bottom two rows of figures) shows that less than 10 % of the added antigen was free and precipitable by its antiserum after such treatment, although only traces of antigen remained in the insoluble residue after extraction with saline–citrate. It seemed possible that some of the antigen might have been degraded during the incubation of the extract with the anti-serum at 37°. This was checked by precipitating with 5 % $HClO_4$ the protein from a sample of the supernatant, obtained after the control ovalbumin–anti-ovalbumin precipitate had been removed, and counting the $HClO_4$ extract. Table 7 shows that less than 10 % of the protein-bound radioactivity in the extract was not

Table 7. *Protein-bound* $^{35}S$ *in plasma and in fractions from spleens of rats after intraperitoneal injection with* $^{35}S$*-labelled anthranilylazo bovine serum albumin*

All counts/min. are corrected to $10^6$ counts/min. injected. AnBSA, Anthranilylazo bovine serum albumin; OA, ovalbumin.

| Time after injection (days) | Counts/min. in saline–citrate extract after treatment with | | Counts/min. precipitated from saline–citrate extract in the presence of | | Counts/min. in residue | Counts/min./ml. of plasma | Counts/min. in saline–citrate extract not precipitated by $HClO_4$ |
|---|---|---|---|---|---|---|---|
| | anti-AnBSA | OA–anti-OA | anti-AnBSA | OA–anti-OA | | | |
| 0·7 | 2370 | 2220 | 29 | 10 | 3855 | 9450 | — |
| 2 | 851 | 653 | 18 | 9 | 1383 | 1570 | — |
| 2 | 775 | 583 | 14 | 0 | 1966 | 1480 | — |
| 4 | 878 | 628 | 12 | 0 | 1258 | — | 1050 |
| 4 | 1228 | 708 | 0 | 0 | 2075 | 1300 | 1273 |
| 5 | 965 | 928 | 3 | 0 | 1755 | 1040 | — |
| 5 | 954 | 1031 | 3 | 0 | 1752 | 1430 | — |
| 7 | 1400 | 757 | 29 | 0 | 1915 | 735 | 1133 |
| 12 | 693 | 615 | 4 | 4 | 1154 | 366 | — |
| 12 | 687 | 694 | 6 | 6 | 1404 | 421 | — |
| 19 | 420 | — | 4 | — | 1095 | 196 | — |
| Addition *in vitro* of $^{35}S$-labelled anthranilylazo bovine serum albumin ($10^4$ counts/min.) to spleens of uninjected rats (see text). | | | | | | | |
| 0 | 9470 | 9170 | 801 | 52 | 268 | — | 790 |
| 0 | 9640 | 8770 | 758 | 60 | 205 | — | 790 |

Table 8. *Protein-bound* $^{35}S$ *in plasma samples and spleens from rats after intraperitoneal injection of* $^{35}S$*-labelled rat serum albumin*

All counts/min. are corrected to $10^6$ counts/min. injected.

| Time after injection (days) | Counts/min. in spleen | Wt. of spleen (g.) | Counts/min./ml. of plasma | Protein-distribution factor* |
|---|---|---|---|---|
| 2 | 2495 | 1·26 | 9930 | 0·26 |
| 2 | 2024 | 0·61 | 9444 | 0·35 |
| 5 | 1869 | 0·92 | 3175 | 0·64 |
| 5 | 2157 | 0·89 | 3510 | 0·66 |
| 8 | 1246 | 1·97 | 685 | 0·92 |
| 11 | 1273 | 0·89 | 408 | 3·51 |
| 11 | 813 | 0·86 | 317 | 2·98 |

* See footnote to Table 1.

Table 9. *Protein-bound* $^{35}S$ *in fractions from spleens of rats after intraperitoneal injection with* $^{35}S$*-labelled rat serum albumin*

All counts/min. are corrected to $10^6$ counts/min. injected. RtSA, Rat serum albumin; BSA, bovine serum albumin.

| Time after injection (days) | Counts/min. extracted by saline–citrate | Counts/min. precipitated from saline–citrate extract in the presence of | | Counts/min. in residue | Counts/min. extracted from residue by $HClO_4$ |
|---|---|---|---|---|---|
| | | Anti-RtSA | BSA–anti-BSA | | |
| 2 | 1385 | 797 | 22 | 1110 | 1164 |
| 2 | 1052 | 614 | 12 | 972 | 759 |
| 5 | 837 | 322 | 34 | 1032 | 735 |
| 5 | 909 | 89 | 24 | 1248 | 777 |
| 8 | 547 | 50 | 33 | 699 | 303 |
| 11 | 628 | 46 | 5 | 645 | 162 |
| 11 | 408 | 73 | 0 | 405 | 228 |

precipitable by $HClO_4$, so that proteolytic degradation can have occurred only to a very limited extent.

*Injection of rat serum albumin.* Finally a series of rats were injected with a homologous protein, rat serum albumin, to compare its behaviour with that of the heterologous antigens. It disappeared from the circulation with a half-life of 1·9 days.

Calculation of the protein-distribution factor of the spleens of these animals (Table 8) shows that it increased fairly rapidly during the 11 days after injection, by which time it is evident that considerable passage of the protein out of the extracellular fluid must have occurred.

Precipitin tests were set up on the saline–citrate extracts of these spleens. Since rat serum albumin was present in high concentration, small volumes of these extracts were taken so that only small volumes of antiserum were required for complete precipitation of the rat serum albumin. Consequently the measured radioactivity in the precipitates was small, and as this measured activity had to be multiplied by a comparatively large factor to express the total radioactivity in the saline–citrate extract, experimental errors are probably larger in this series than in the others. The results, in Table 9, show that about 60 % of the protein-bound $^{35}S$ in the extracts was precipitable by anti-rat serum albumin 2 days after injection, but that this percentage declined fairly rapidly during the subsequent 9 days.

In a search for possible degradation products of the injected rat serum albumin, $HClO_4$ extracts were made on the residues of the spleen, and Table 9 shows that considerable amounts of radioactivity were found in these extracts.

## DISCUSSION

*Injection of rat serum albumin.* In order to compare the results observed after the injection of heterologous proteins, some experiments were done with rat serum albumin. This homologous protein appears to leave the extracellular-fluid compartment of the spleen fairly rapidly, and progressively larger amounts of it appear in a form which is no longer precipitable by its antiserum. This could be due to the combination of the rat serum albumin with some substance in the spleen which masks the antigenically determinant groups, or to degradation of the molecules into fragments which are too small to precipitate with antisera to rat serum albumin, but which are still big enough to be precipitated by methanol in the procedure used for measuring protein-bound $^{35}S$. Considerable degradation of the rat serum albumin may have occurred since perchloric acid extracted $^{35}S$ from the residues remaining after the spleens had been extracted with saline–citrate.

It was hoped that the data obtained after the injection of rat serum albumin would allow some estimate of the extracellular-fluid volume of the spleen, but in view of the fairly rapid passage of rat serum albumin (or its breakdown products) out of the extracellular fluid only a maximum value can be set. If the protein-distribution factor of the two spleens of the rats killed 2 days after injection are averaged, a value of 0·28 ml./g. of spleen is obtained. This seems of the right order of magnitude for the extracellular-fluid volume.

*Injection of bovine γ-globulin.* Injected bovine γ-globulin left the plasma rapidly at an exponential rate, but it is possible that there may have been immune elimination of some of the antigen from the plasma of the rats killed 8 and 10 days after injection. The radioactivity in the plasmas of these rats was less than one-tenth of the amount that would have been expected if there had been no immune elimination (see Fig. 1). The amounts of protein powder obtained after processing the plasma samples of these rats were very similar to those obtained from plasma samples of the other rats, and there was no reason to suspect any faults in the radioactivity measurements. Therefore these results are believed to be reliable, even though there is no evidence of immune elimination of any of the antigen from the plasmas of the rats killed 12 and 18 days after injection. The only explanation that can be offered for this discrepancy is that there may be considerable variation between the animals used. [Weigle & Dixon (1957) observed immune elimination in only five out of eight rats injected with rabbit γ-globulin.]

During the 7 days after injection most of the radioactivity extractable with saline–citrate was precipitable by its antiserum, and is therefore considered to be bovine γ-globulin, substantially unaltered in its determinant groups. However, at this time, when immune elimination of antigen from the plasma may have been occurring in some rats, there was an abrupt change in the properties of the saline–citrate extracts. In rats killed at later times no significant amounts of radioactivity were precipitated when the saline–citrate extracts were treated with anti-bovine γ-globulin. There is evidence that some of the antigen (or its breakdown products) in the spleen had passed out of the extracellular-fluid compartment in most of the rats killed 8 days after injection and later, a phenomenon which is known to occur in rabbits which are in the immune state (Francis *et al.* 1957). There may be some causal relation between these phenomena. If antibody production started on the eighth day after injection it might have been expected that antigen already in the spleen would have reacted with newly formed antibody there and thus become insoluble in saline–citrate. However, there is only a very slight

rise in the proportion of radioactivity left unextracted by saline–citrate after the eighth day. Thus formation of a complex with some other type of substance yielding a soluble product, or degradation to products no longer capable of precipitating with antibody, seems a more likely fate for the antigen. The formation of a complex is suggested by the fact that, after removal of the free bovine $\gamma$-globulin from the saline–citrate extracts by precipitation with anti-bovine $\gamma$-globulin, considerable amounts of $^{35}S$ were precipitated when the extracts were brought to pH 4·5. The rats killed 8 and 10 days after injection may have been immune, and in extracts from their spleens a higher proportion of the radioactivity was precipitated than from spleen extracts of rats killed 3·5 days after injection. These latter rats showed no evidence of being immune. Such a method of fractionation is, of course, non-specific: Kay & Dounce (1953) used it to precipitate ribonucleoprotein from saline extracts of animal tissues. We found that over 95% of the alkali-labile phosphate in the extract occurs in this fraction. The pH 4·5 precipitate therefore contains substantially all the ribonucleic acid of the extract, to which, according to Garvey & Campbell (1957), some of the antigen might be bound. It is also likely that soluble complexes between bovine $\gamma$-globulin or its breakdown products and antibodies to bovine $\gamma$-globulin would be precipitated in this fraction.

Investigation of the deoxyribonucleoprotein fraction obtained from the spleens by extraction with water showed only a small amount of radioactivity, and as this disappeared from this fraction on further purification it is concluded that no antigen can be associated with deoxyribonucleoprotein.

In all the spleens investigated a small proportion of the radioactivity remained unextractable by saline–citrate and water. There is a tendency for the proportion of radioactivity in this fraction to increase with time. It is probably due to complexes of the antigen (or its breakdown products) with some material in the cells, such as ribonucleic acids, mucopolysaccharides or lipids.

*Injection of bovine serum albumin.* After injection of bovine serum albumin a similar series of events seems to take place, though the time-scale is somewhat shortened. The percentage of the extractable $^{35}S$ that is specifically precipitable by anti-bovine serum albumin falls steadily from about 60% on the first day after injection to a barely significant figure by the fourth and fifth days. The passage of antigen and its breakdown products out of the extracellular fluid is evident on the third day after injection and thus occurs more rapidly than after injection of bovine $\gamma$-globulin. This may be a reflexion of the smaller size of the bovine serum albumin molecule. Only two animals (killed on the third and ninth days) showed any evidence of immune elimination of the antigen from the plasma.

Some evidence of complex formation was found by precipitation of saline–citrate extracts at pH 4·5. In the spleens of rats killed 1 and 2 days after injection much of the $^{35}S$ in the saline–citrate extracts was precipitable by anti-bovine serum albumin and no antigen had passed out of the extracellular fluid. The precipitates which were formed at pH 4·5 from these rats' spleens contained less $^{35}S$ than those obtained from spleen extracts of rats killed at later times, when less antigen was precipitable by anti-bovine serum albumin and where considerable passage of the antigen out of the extracellular fluid may have occurred.

An appreciable amount of radioactivity remained unextracted by the solvents used. In some cases portions of this residue were treated with trichloroacetic acid and then washed with acetone, and the radioactivity of the residue was determined in the normal way. This procedure might be expected to dissociate any bovine serum albumin in a loose complex, because of the denaturing action of the trichloroacetic acid, and if this occurred the trichloroacetate of bovine serum albumin should dissolve in the acetone (Levine, 1954), leaving behind bovine serum albumin that is probably in the form of a firm complex in insoluble materials. Since very little of the radioactivity is extracted by this procedure, the presence of such complexes is indicated.

Several attempts were made to digest the ribonucleic acid in the saline–citrate extracts of the spleens of animals injected both with bovine $\gamma$-globulin and with bovine serum albumin, so that if any of the antigen had been conjugated with ribonucleic acid it should have been set free and would have precipitated on addition of the appropriate antiserum. Dialysis at pH 10 for 16 hr. (cf. Fraenkel-Conrat & Williams, 1955) or digestion with ribonuclease in the presence and absence of 8M-urea and 4M-guanidine was unsuccessful in liberating immunologically active antigen.

*Injection of anthranilylazo bovine serum albumin.* Anthranilylazo bovine serum albumin seemed to suffer a fate different from that of the two native antigens. This is in accordance with the known behaviour of extensively modified proteins *in vivo* (Dixon *et al.* 1951; Haurowitz & Crampton, 1952; Francis & Hawkins, 1957). It disappeared very rapidly from the plasma and greater amounts of $^{35}S$ appeared in the spleen than after the injection of native proteins. However, even less than 1 day after injection, only negligible amounts of $^{35}S$ were precipitated from the saline–citrate extracts on addition of anti-anthranilylazo bovine serum albumin. Thus it is unlikely that any of the radioactivity in the spleen was in the form of free antigen capable of precipitating with its antibody.

The calculation of the amount of protein-bound radioactivity in the saline–citrate extracts was uncertain. In some cases considerably more protein-bound $^{35}S$ was found in the saline–citrate extracts after treatment with anti-anthranilylazo bovine serum albumin than after treatment with ovalbumin–anti-ovalbumin. A possible explanation for this discrepancy is that there may have been some breakdown products of the anthranilylazo bovine serum albumin in the extract which would combine with antibodies to anthranilylazo bovine serum albumin without precipitating. These complexes would probably be precipitated by the methanol–acetate procedure used. After precipitation of the saline–citrate extracts of some of these spleens with perchloric acid, considerable amounts of radioactive material remained soluble. This indicates the presence of breakdown products derived from the antigen.

When anthranilylazo bovine serum albumin was added to homogenates of two normal spleens, very little was precipitated by anti-anthranilylazo bovine serum albumin. Since very little proteolytic degradation had occurred, this strongly suggests that there may be substances in the spleen with which anthranilylazo bovine serum albumin can form a complex very readily so as to render it unavailable for precipitation with antibody. The presence of such substances in the spleen (and probably elsewhere in the body) which can react with anthranilylazo bovine serum albumin and other extensively modified proteins would explain the very rapid disappearance of these proteins from the blood stream and their retention in the tissues. Such a possibility has already been envisaged by Crampton *et al.* (1953).

*General conclusions.* The experiments reported here have shown that free (i.e. available for precipitation by homologous antibody) native-protein antigens can be detected in rat spleens up to about a week after injection. Haurowitz (1953) has suggested that only very small amounts of antigen are necessary for immunization, and the free antigen that has been detected in spleens may be part of the excess of antigen that plays no immediate part in the induction of antibody formation. In some cases free antigen had disappeared from the spleen by the time that changes associated with the immune state were apparent, although there were still considerable amounts of protein-bound $^{35}S$ in the spleen. Possibly a necessary stage in the development of immunity is the formation of complexes of antigen with other substances in the cells of the host. Garvey & Campbell (1957) have found radioactive material associated with substances which had an absorption maximum similar to that of nucleic acid in the liver of rabbits after injection of radioactive bovine serum albumin. In view of the association of ribonucleic acid with protein synthesis, complexes of antigen with ribonucleic acid might be expected to play a part in the production of antibodies. Though we have shown that protein-bound $^{35}S$ occurs in the same fraction of spleen as ribonucleic acid, attempts to demonstrate definite conjugation of antigen with ribonucleic acid have so far been unsuccessful.

## SUMMARY

1. Bovine $\gamma$-globulin, bovine serum albumin and rat serum albumin trace-labelled with diazotized [$^{35}S$]sulphanilic acid have been injected into rats and their spleens removed at various times up to 18 days after injection.

2. The persistence of these proteins in the plasma has been followed. Bovine $\gamma$-globulin has a half-life of 2·5 days, bovine serum albumin of 2·0 days and rat serum albumin of 1·9 days.

3. Bovine $\gamma$-globulin and bovine serum albumin precipitable by their homologous antibodies have been found in saline extracts of the spleen up to 7 days after injection of bovine $\gamma$-globulin and up to 4 days after injection of bovine serum albumin.

4. Some of the protein-bound $^{35}S$ in the spleen has been found in the same fraction as the ribonucleic acid, in agreement with the views of Garvey & Campbell (1957).

5. After injection of labelled anthranilylazo bovine serum albumin, saline extracts of the spleen contain protein-bound $^{35}S$ but none of this is precipitable by anti-anthranilylazo bovine serum albumin. Similarly, less than 10% of anthranilylazo bovine serum albumin added *in vitro* to spleen homogenates is available for precipitation by anti-anthranilylazo bovine serum albumin.

6. Rat serum albumin injected for comparison with the heterologous proteins has been recovered from spleen extracts in a form precipitable by its antiserum only up to 5 days after injection.

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# Possible Sites of Catabolism of Rat Serum Albumin

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Many studies have been made of the turnover of mammalian serum proteins, generally with isotopically labelled proteins, but very little information is available about the sites of breakdown. Cohen & Gordon (1958) have demonstrated the catabolism of rat serum albumin by the perfused liver and have concluded that this organ can catabolize only about 13–15% of the protein which is broken down in the intact animal.

In the course of experiments reported in the preceding paper (Hawkins & Haurowitz, 1961), rats were killed at various times after the injection of rat serum albumin, trace-labelled with [$^{35}$S]-sulphanilic acid, and the spleens were removed. At the same time other organs were taken out and their content of protein-bound $^{35}$S and $^{35}$S extractable with perchloric acid was determined. These observations have permitted some tentative conclusions to be drawn about the sites of protein catabolism, and they form the subject of this paper.

## MATERIALS AND METHODS

The preparation of rat serum albumin, trace-labelled with [$^{35}$S]sulphanilic acid, is described by Hawkins & Haurowitz (1961). A portion (3·0 mg.) of this preparation was injected intraperitoneally into seven rats, which were killed at various times up to 11 days later. Blood (about 3 ml.) was obtained in an oxalated syringe by cardiac puncture and the plasma separated by centrifuging. Then the spleen, lungs, liver, kidneys, a sample of muscle from the abdominal wall, and the inguinal lymph nodes, were rapidly excised, weighed and all except the spleens frozen at −15° until they could be used. The spleens were treated immediately as described by Hawkins & Haurowitz (1961). The other tissues were treated similarly. They were thawed, chopped finely with scissors and homogenized with five times their weight of cooled 0·10M-NaCl–0·05M-sodium citrate (saline–citrate; Chargaff, 1955). The homogenates were centrifuged at 10 000 *g* for 20 min. at 0° and the residues re-extracted with saline–citrate (three times the organ weight) and centrifuged again. The extracts were combined and their total volume was measured. The residues were evenly suspended in 5·0 ml. of saline–citrate. A measured portion of each of the tissue extracts and residues and plasma samples was extracted by adding 2 vol. of 10% (w/v) $HClO_4$, centrifuging and re-extracting the residue twice with 1 vol. of 7% $HClO_4$. The extracts were combined and made up to a convenient volume for the determination of radioactivity. The precipitated protein was suspended in 5 ml. of water and the protein-bound $^{35}$S in this fraction determined by the methanol–acetate procedure (Hawkins & Haurowitz, 1961).

*Determination of radioactivity.* Protein-bound $^{35}$S was measured by weighing samples (30±0·2 mg.) of the dried and finely powdered protein on nickel-plated planchets and counting in a windowless Geiger counter (Banks, Blow & Francis, 1956). $^{35}$S in the $HClO_4$ extracts was determined by Steinberg's (1959) method. Portions (3·0 ml.) of the extracts were pipetted on to 1·0 g. of finely ground *p*-terphenyl (L. Light and Co. Ltd.) in a glass dish and counted with a scintillation counter. A drop of the neutral detergent Stergene was added to enable the $HClO_4$ extract to wet the

hydrophobic *p*-terphenyl. Under the conditions used the background count was about 30 counts/min. Sample counts of less than 10 counts/min. above background have been recorded as not significant. The total radioactivity of the protein injected as determined by this method was about $7 \times 10^5$ counts/min.; as determined with the windowless Geiger counter it was slightly lower. To facilitate comparison between radioactivity determinations in the different counters, all counts/min. have been corrected to an arbitrary figure of $10^6$ counts/min. injected.

The protein-distribution factor for each organ was calculated as described by Hawkins & Haurowitz (1961). This is equal to (counts/min. protein-bound $^{35}S$/g. of tissue)/(counts/min. protein-bound $^{35}S$/ml. of plasma). As explained there, this is useful for comparing results from tissues of different weights.

## RESULTS AND DISCUSSION

As discussed by Hawkins & Haurowitz (1961), the protein-distribution factor gives a measure of the volume of the extracellular-fluid compartment of an organ, provided that all the radioactive protein stays in the extracellular fluid. In general, the protein-distribution factors of the tissues investigated tended to increase as the time after injection increased, so the values of this factor observed 2 days after injection may give the volume of the extracellular-fluid compartment, or they may overestimate it. There are several reasons why the protein-distribution factor may have a high value. In the present work, the existence of a high value has been taken to mean that the protein in the tissue has passed out of the extracellular-fluid compartment in some way. This is a very likely prerequisite for catabolism to take place, but it does not prove that the tissue in question can catabolize the protein.

Only very small amounts of non-protein-bound $^{35}S$ were found in the plasma 5 days and more after injection (see Table 1). Thus any appreciable amount of non-protein-bound $^{35}S$ found in the saline–citrate extract of a particular tissue at these times cannot have come solely from the plasma trapped in the tissue, but was probably a product of catabolism of the injected protein by that tissue.

In the present work perchloric acid has been used to precipitate protein and to extract soluble degradation products. Any method of protein precipitation must be somewhat arbitrary since a search is being made for degradation products of a protein and the larger ones will probably have properties very similar to those of the injected protein. Treatment with perchloric acid is a widely used procedure for precipitating proteins and separating them from non-protein material, but the upper size limit (if indeed size is the main determining factor) of peptides derived from degraded proteins that will still be extracted by perchloric acid is unknown. It is quite likely therefore that extraction with perchloric acid may slightly under-estimate the degree of degradation of protein, but it must give some indication of the amount of low-molecular-weight material produced in this process.

*Plasma.* Table 1 shows that there was some $^{35}S$ extractable from the plasma by perchloric acid 2 days after injection. This probably consists of breakdown products of the injected protein which have diffused out of various organs where catabolism has taken place. At later times the amount of this perchloric acid-extractable $^{35}S$ decreased until there was none in the plasma. It would be expected that catabolism would proceed at a higher rate at short times after injection when there was a relatively high concentration of the injected protein in the body. The protein-bound $^{35}S$ disappeared from the plasma at an exponential rate, with a half-life of 1·9 days. Weigle & Dixon (1957) reported that injected rabbit serum albumin disappeared very rapidly from the blood of rats.

*Spleen.* As this organ was required for other purposes, it was not possible to extract the saline–citrate extracts with perchloric acid. The protein-bound $^{35}S$ in saline–citrate extracts of this organ was therefore determined by precipitation with methanol–acetate. Table 2 shows that considerable amounts of protein-bound $^{35}S$ had penetrated out of the extracellular-fluid compartment by 5 days after injection. At all times after injection only about half the protein-bound $^{35}S$ in the organ was extractable with saline–citrate. The protein-bound $^{35}S$ insoluble in saline–citrate is probably in the form of fairly firm complexes between the rat serum albumin and some tissue components. As the absolute amount of $^{35}S$ insoluble in saline–citrate decreases with time, it is possible that these complexes represent an intermediate stage in the catabolism of the protein. The occurrence of considerable amounts of $^{35}S$ extractable by perchloric acid from the insoluble residue strongly suggests that catabolism of the protein occurs in the spleen.

Table 1. *Radioactivity of rat plasma after injection of rat serum albumin trace-labelled with* $^{35}S$

All counts/min. are corrected to $10^6$ counts/min. injected.

| Time after injection (days) | Protein-bound $^{35}S$ (counts/min./ml.) | $^{35}S$ extracted by $HClO_4$ (counts/min./ml.) |
|---|---|---|
| 2 | 9930 | 238 |
| 2 | 9444 | 226 |
| 5 | 3175 | 88 |
| 5 | 3510 | 76 |
| 8 | 685 | 34 |
| 11 | 408 | ns* |
| 11 | 317 | ns |

* Not significant; less than 10 counts/min. above background.

Table 2. *Radioactivity of spleen fractions after injection of rat serum albumin trace-labelled with* $^{35}S$

All counts/min. are corrected to $10^6$ counts/min. injected.

| Time after injection (days) | Wt. of spleen (g.) | Protein-bound $^{35}S$ in: Saline–citrate extract (counts/min.) | Protein-bound $^{35}S$ in: Residue (counts/min.) | Protein-distribution factor* | $^{35}S$ extracted from residue by $HClO_4$ (counts/min.) |
|---|---|---|---|---|---|
| 2 | 1·26 | 1385 | 1110 | 0·26 | 1160 |
| 2 | 0·61 | 1052 | 972 | 0·35 | 760 |
| 5 | 0·92 | 837 | 1032 | 0·64 | 730 |
| 5 | 0·89 | 909 | 1248 | 0·66 | 780 |
| 8 | 1·97 | 547 | 699 | 0·92 | 300 |
| 11 | 0·89 | 628 | 645 | 3·51 | 160 |
| 11 | 0·86 | 408 | 405 | 2·98 | 230 |

* See Materials and Methods section.

Table 3. *Radioactivity of tissue fractions after injection of rat serum albumin trace-labelled with* $^{35}S$

All counts/min. are corrected to $10^6$ counts/min. injected.

| Time after injection (days) | Wt. of tissue (g.) | Protein-bound $^{35}S$ in: Saline-citrate extract (counts/min.) | Protein-bound $^{35}S$ in: Residue (counts/min.) | Protein-distribution factor* | $^{35}S$ extracted by $HClO_4$ from: Saline–citrate extract (counts/min.) | $^{35}S$ extracted by $HClO_4$ from: Residue (counts/min.) |
|---|---|---|---|---|---|---|
| | | | Liver | | | |
| 2 | 10·2 | 11 350 | 1960 | 0·13 | 14 900 | 2955 |
| 2 | 8·1 | 10 120 | 1700 | 0·15 | 12 900 | 1040 |
| 5 | 9·4 | 6 270 | 1346 | 0·25 | 5 590 | 1010 |
| 5 | 9·1 | 5 650 | 1320 | 0·22 | 6 460 | 675 |
| 8 | 17·2 | 1 828 | 735 | 0·22 | 940 | 0 |
| 11 | 11·8 | 3 430 | 1025 | 0·92 | 2 490 | 500 |
| 11 | 10·4 | 1 800 | 763 | 0·78 | 1 830 | 380 |
| | | | Kidney | | | |
| 2 | 1·86 | 4 980 | 705 | 0·30 | 9 195 | 410 |
| 2 | 1·87 | 5 130 | 972 | 0·34 | 10 170 | 1120 |
| 5 | 1·92 | 3 660 | 777 | 0·73 | 7 870 | 620 |
| 5 | 2·00 | 4 030 | 846 | 0·70 | 7 250 | 750 |
| 8 | 3·03 | 2 260 | 498 | 1·33 | 3 050 | 200 |
| 11 | 2·32 | 2 835 | 927 | 3·97 | 2 960 | 500 |
| 11 | 2·32 | 1 523 | 957 | 3·37 | 2 790 | 370 |
| | | | Lung | | | |
| 2 | 1·20 | 2 645 | 93 | 0·23 | 230 | 0 |
| 2 | 1·11 | 2 260 | 201 | 0·23 | 280 | 0 |
| 5 | 1·49 | 1 326 | 128 | 0·31 | 456 | ns† |
| 5 | 1·34 | 993 | 66 | 0·23 | 180 | ns |
| 8 | 1·58 | 350 | 81 | 0·40 | ns | 0 |
| 11 | 1·25 | 154 | 36 | 0·37 | ns | ns |
| 11 | 1·30 | 195 | 54 | 0·54 | 420 | ns |
| | | | Skeletal muscle | | | |
| 2 | 1·60 | 927 | 234 | 0·07 | 266 | ns |
| 5 | 1·93 | 423 | 111 | 0·09 | 320 | ns |
| 5 | 2·02 | 403 | 30 | 0·06 | ns | 0 |
| 8 | 1·47 | 90 | 27 | 0·12 | ns | 0 |
| 11 | 1·84 | 23 | 0 | 0·03 | ns | ns |
| 11 | 1·22 | 36 | 17 | 0·14 | ns | ns |
| | | | Lymph node | | | |
| 2 | 0·85 | 1 665 | 60 | 0·20 | 445 | 0 |
| 2 | 0·30 | 662 | 20 | 0·24 | 162 | 0 |
| 5 | 0·77 | 742 | 30 | 0·32 | 277 | 0 |
| 5 | 0·62 | 604 | 25 | 0·29 | 191 | 0 |
| 8 | 1·06 | 287 | 0 | 0·39 | 200 | ns |
| 11 | 0·64 | 95 | 0 | 0·36 | 128 | ns |
| 11 | 0·78 | 166 | 0 | 0·67 | 187 | ns |

* See Materials and Methods section. † Not significant (less than 10 counts/min. above background).

*Liver.* As might be expected from its large size and high metabolic activity, large amounts of $^{35}S$ were found in this organ (Table 3). Since the proportion of protein-bound $^{35}S$ insoluble in saline–citrate was comparatively low, complexes between the protein and tissue components are not likely to be present to any great extent in this organ. This is also suggested by the figures for the protein-distribution factor, which were much lower than those for the spleen, despite the fact that both organs have sinusoidal vascular systems and are presumably fairly rich in blood. However, there is definite evidence that some protein-bound $^{35}S$ had left the extracellular-fluid compartment 11 days after injection, and this may possibly have happened earlier. Breakdown products from the injected protein were extractable by perchloric acid from the saline–citrate extracts in considerable quantities throughout the period of observation and small amounts were also usually extractable from the insoluble residue. These findings support the claim of Cohen & Gordon (1958) that the liver catabolizes serum albumin.

*Kidney.* Next to the liver, this organ contained the greatest amount of protein-bound $^{35}S$ of any organ studied (Table 3), and the concentration of $^{35}S$ was a good deal higher than in the liver. All of the protein-breakdown products formed in the animal have to pass through the kidney if they are excreted in the urine, but this would hardly account for the large amount of protein-bound $^{35}S$, most of which would be in a form not likely to be excreted by this route. The high values of the protein-distribution factor suggest that a good deal of protein-bound $^{35}S$ has left the extracellular-fluid compartment. Compared with the spleen, only a small proportion of this protein-bound $^{35}S$ appears to have formed complexes with insoluble tissue constituents, since comparatively little protein-bound $^{35}S$ was unextractable by saline–citrate. The large amounts of $^{35}S$ extractable by perchloric acid from the saline–citrate extracts could be breakdown products of the protein, arising by catabolism elsewhere, or they could have arisen by degradation of the protein in the kidney. Because of the large amount of protein-bound $^{35}S$ present, it seems likely that some of the extractable $^{35}S$ is in the latter class and therefore that this organ is a site of catabolism of serum albumin.

*Lungs.* Most of the protein-bound $^{35}S$ of the lungs was extractable by saline–citrate throughout the experiment (Table 3). The low values for the protein-distribution factor, which only began to rise appreciably at 8 days, suggest that little protein passed out of the extracellular-fluid compartment. Only small amounts of $^{35}S$ were extractable by perchloric acid from the saline–citrate extracts, so that it is concluded that this organ can catabolize serum albumin only to a limited extent, if at all.

*Skeletal muscle.* As shown in Table 3, most of the values for the protein-distribution factor were low. It is not known whether this is due to a low content of extracellular fluid in muscle or to slow equilibration between this extracellular fluid and the plasma. The higher values observed in two cases may have been due to slight contamination with blood, even though the pieces of tissue were carefully blotted to remove adherent blood immediately after removal from the animal. The protein-bound $^{35}S$ in the tissue was nearly all extractable by saline–citrate, and the small amounts of $^{35}S$ extractable by perchloric acid from the saline–citrate extracts of two pieces of muscle were on the lower limit of detection. As might be expected from its highly specialized function, catabolic activity of this tissue towards serum albumin was not demonstrable.

*Lymph nodes.* Lymphatic tissue was included in this survey since it may well play a part in the degradation of heterologous proteins, aided perhaps by the immune response of the host animal. When the lymph nodes were removed from the animals no attempt was made to free them completely from the fat in which they were embedded. After homogenization and centrifuging, the fat floated to the top of the tube as a solid white mass, which was removed, weighed and the weight subtracted from the total weight of the tissue. The weights in Table 3 therefore refer to the weights of the nodes themselves.

The lymph nodes seem to possess only very limited catabolic activity with respect to homologous serum albumin, judging by the very small extent to which protein-bound $^{35}S$ passed out of the extracellular-fluid compartment. Barely detectable amounts of protein-bound $^{35}S$ were unextractable by saline–citrate and there were only small quantities of $^{35}S$ extracted by perchloric acid from the saline–citrate extracts.

## SUMMARY

1. Plasma, spleen, liver, kidneys, lungs, lymph nodes and samples of skeletal muscle from rats have been analysed for protein-bound $^{35}S$ and $^{35}S$ extractable with perchloric acid at various times after intraperitoneal injection of rat serum albumin, trace-labelled with $^{35}S$.

2. From the observed distribution of protein-bound $^{35}S$ and $^{35}S$ extractable with perchloric acid it is suggested that spleen, liver and kidneys all catabolize homologous serum albumin. Lungs and lymph nodes may exhibit very limited catabolic activity, whereas skeletal muscle has no such activity.

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# Uptake of Pyrithiamine by Tissue of Rats

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Although the thiamine and thiamine diphosphate content of the tissues of animals treated with pyrithiamine, 1-[(4-amino-2-methyl)pyrimid-5-ylmethyl]-2-methyl-3-(β-hydroxyethyl)pyrimidinium bromide hydrobromide, has been studied, no data are available regarding the pyrithiamine content that may be related to thiamine levels, and that might clarify the mechanism producing avitaminosis $B_1$ in animals treated with pyrithiamine. Administration of pyrithiamine causes a decrease of the total thiamine and thiamine diphosphate content of all the tissues, particularly of the brain, in mice and rats (De Caro, Rindi & Grana, 1954; De Caro, Rindi, Perri & Ferrari, 1956*a*, *b*, 1958; De Caro, Rindi & Ferrari, 1958; Koedam & Steyn-Parvè, 1959), and in pigeons (Koedam, Steyn-Parvè & van Rheenen, 1956; Koedam, 1958). The diminution in the brain is both marked and early, whereas in the alimentary thiamine deficiency the brain more firmly preserves its thiamine content (Salcedo, Najjar, Holt & Hultzer, 1948; Rindi, Ferrari & Perri, 1954).

The present paper describes a microfluorimetric method for determining pyrithiamine in rat tissues and reports the results obtained in animals treated with pyrithiamine, with and without thiamine.

## EXPERIMENTAL

*Materials.* Adsorbent charcoal was prepared by treatment of activated charcoal (50 SL, produced by Italian C.E.C.A., Milan) with cholesteryl stearate (British Drug Houses Ltd.), as described by Siliprandi & Siliprandi (1954). Columns were prepared according to Rindi & de Giuseppe (1961). Amberlite IRC-50 resin (British Drug Houses Ltd., CG-50) was utilized in a carboxylic form after treatment with 2N-HCl, followed by washing with water until disappearance of the $Cl^-$ reaction. Pyrithiamine was a commercial sample of Neopyrithiamine hydrobromide purchased from the California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A. The mono- and di-phosphate were prepared by the method of Viscontini, Bonetti & Karrer (1951) and purified on ion-exchange resin. Cellulose powder (Whatman, ash-free cellulose) was washed with 50% (v/v) ethanol, filtered on a Buchner funnel, thoroughly rinsed with water and dried.

*Determination of pyrithiamine.* The rapidly excised tissue (liver, brain, kidney, 3–5 g.; muscle, 9–11 g.) was weighed and homogenized in 2·5 vol. of 0·5M-$HClO_4$ in a MSE homogenizer. After centrifuging (3500 *g*), the clear supernatant fluid was separated and the residue again homogenized in 1 vol. of 0·5M-$HClO_4$ and centrifuged. The combined supernatant fluids were neutralized (pH 6·8–6·9) with 4M-KOH. After 20 min. at 0°, the precipitated $KClO_4$ was removed by centrifuging in a refrigerated centrifuge. To the clear extract, at room temperature and adjusted to pH 4·5 with 50% (v/v) acetic acid, 1 ml. of a freshly prepared aqueous suspension (100 mg./ml.) of Taka-diastase (Parke Davis and Co. Ltd.) was added. The mixture was incubated overnight at 37°. The solution, adjusted to pH 6·8–6·9 with NaOH, was then passed through a column (1·2 cm. diam.) of charcoal–cellulose powder (1:5); 200 mg. of charcoal for liver, brain and kidney, 300 mg. for muscle. The flow rate was 6–8 drops/min. The column was washed with 30 ml. of water and eluted with 25 ml. of 10% (v/v) propan-1-ol (Rindi & de Giuseppe, 1961). The eluate was passed through a column (3 cm. × 0·9 cm.) of Amberlite IRC-50. The flow rate was 10–12 drops/min. The column was washed with 20 ml. of water and eluted with about 25 ml. of 0·1N-HCl. The eluate was made up to 25 ml. with the eluent, if necessary. Solid KCl (400 mg.) was dissolved in each of two samples of 1–2 ml. of eluate, adjusted to 2 ml. with water, in stoppered centrifuge tubes; then 0·5 ml. of 10N-NaOH was added to each. The samples were incubated for 60 min. at 37° to destroy the thiamine and 0·5 ml. of 10N-NaOH was added to one tube (B) and 0·5 ml. of 0·20% (w/v) $K_3Fe(CN)_6$ in

10 N-NaOH (cf. Sealock & White, 1949) to the other (A). After 4 min. the solutions were extracted by shaking for 90 sec. with 5 ml. of *iso*butanol. The aqueous layer was removed after centrifuging, and the *iso*butanol was dehydrated by addition of 0·5 g. of anhydrous $Na_2SO_4$ and centrifuged. The fluorimetric measurement was performed on 1 ml. of the dried *iso*butanol layer in a model A Farrand microfluorimeter, and compared with a quinine standard (20 $\mu$mg./ml. of 0·1 N-$H_2SO_4$). The difference between the fluorescence values of A and B gave the fluorescence corresponding to pyrithiamine. A calibration curve, which was linear over the range 0–9 $\mu$g., was obtained from an aqueous solution of pyrithiamine treated as above, beginning with chromatography on Amberlite resin. The addition of 3 $\mu$g. of thiamine caused no significant interference. Under the above conditions a fluorescence value was obtained with tissues from rats which had not been treated with pyrithiamine. These values have been subtracted from the values obtained for tissues of treated rats. The values corresponded to a pyrithiamine value ($\mu$g./g. of wet tissue) of about 0·27 in brain, 0·97 in kidney, 1·76 in liver and 0·67 in muscle.

*Phosphorylated pyrithiamine.* The $HClO_4$ extract of the tissue, after removal of excess of $HClO_4$, was divided into two parts. Fraction A, brought to pH 5·2 with 50% (v/v) acetic acid, was treated with 10 mg. of freeze-dried acid phosphatase prepared from human prostate according to Vescia & Testi (1958). This fraction was then incubated for 8 hr. at 37°, while fraction B, brought to pH 5·2, was stored for the same time in a refrigerator. Both fractions were successively brought to pH 6·8 and treated as described for the determination of pyrithiamine. The intensity of fluorescence of fraction A corresponded to 'total pyrithiamine' (free + phosphorylated) and that of fraction B to 'free pyrithiamine'. Pyrithiamine mono- and di-phosphate give a fluorescent oxidation product insoluble in *iso*butanol.

*Animals.* Male albino rats, weighing 85–100 g., placed in single small cages, were reared on a synthetic thiamine-deficient diet, consisting of (%): washed, fat-free casein, 20; wheat starch, 63; olive oil, 10; cod-liver oil, 2; Osborne & Mendel's (1912) salt mixture, 5. The diet was supplemented with suitable amounts of all the B vitamins (except thiamine), administered daily by mouth in aqueous solution.

## RESULTS

Table 1 summarizes the results obtained in experiments on rat tissues removed 24 hr. after the intraperitoneal injection of 1 mg. of pyrithiamine, to which various amounts of pyrithiamine were added during perchloric acid extraction. The recovery ranged from 96·8 to 102·5%.

Tables 2 and 3 show average changes of pyrithiamine content of tissues found after administration

Table 1. *Recovery of pyrithiamine added to some rat tissues*

Tissues were taken from rats 24 hr. after the intraperitoneal injection of 1 mg. of pyrithiamine. Results are given as means ± S.E. for 4 determinations.

| | Pyrithiamine ($\mu$g./g. of wet tissue) | | | Recovery (%) |
|---|---|---|---|---|
| | Present | Added | Found | |
| Brain | 1·42 ± 0·16 | 2·33 ± 0·44 | 3·70 ± 0·44 | 97·6 ± 4·03 |
| Kidney | 2·41 ± 0·33 | 2·57 ± 0·32 | 4·86 ± 0·27 | 97·5 ± 4·57 |
| Liver | 4·25 ± 0·22 | 2·44 ± 0·14 | 6·87 ± 0·36 | 102·5 ± 4·83 |
| Muscle | 0·51 ± 0·02 | 0·76 ± 0·06 | 1·24 ± 0·10 | 96·8 ± 3·38 |

Table 2. *Pyrithiamine content of rat tissues after a single intraperitoneal injection of 1 mg. of pyrithiamine*

The dose was given in 0·2 ml. of 0·9% NaCl. Each value is the average of 7 determinations ± S.E.

| | Pyrithiamine ($\mu$g./g. of wet tissue) | | | | |
|---|---|---|---|---|---|
| Days after dose ... | 1 | 3 | 5 | 9 | 12 |
| Brain | 1·13 ± 0·12 | 1·41 ± 0·21 | 2·13 ± 0·12 | 2·25 ± 0·23 | 2·77 ± 0·17 |
| Kidney | 2·65 ± 0·38 | 1·57 ± 0·28 | 1·18 ± 0·15 | 0·98 ± 0·40 | 0·71 ± 0·24 |
| Liver | 7·74 ± 0·78 | 5·13 ± 0·74 | 4·75 ± 0·65 | 2·58 ± 0·62 | 1·07 ± 0·29 |
| Muscle | 1·45 ± 0·15 | 0·79 ± 0·18 | 0·44 ± 0·05 | 0·46 ± 0·08 | 0·27 ± 0·07 |

Table 3. *Pyrithiamine content of tissues in rats given a daily oral dose of 33 $\mu$g. of thiamine + 210 $\mu$g. of pyrithiamine*

Each value is the average of 6 determinations ± S.E.

| | Pyrithiamine ($\mu$g./g. of wet tissue) | | |
|---|---|---|---|
| Day of treatment ... | 5 | 12 | 20 |
| Brain | 0·91 ± 0·09 | 1·62 ± 0·07 | 3·51 ± 0·20 |
| Kidney | 1·54 ± 0·19 | 2·49 ± 0·19 | 6·49 ± 0·73 |
| Liver | 2·52 ± 0·26 | 4·63 ± 0·48 | 9·52 ± 0·80 |
| Muscle | 0·44 ± 0·12 | 0·86 ± 0·08 | 1·77 ± 0·19 |

of pyrithiamine. The livers of rats which had been injected 24 hr. previously with 1 mg. of pyrithiamine were examined for phosphorylated pyrithiamine. The mean values ± S.E. for 7 determinations of pyrithiamine before and after phosphatase treatment were 0·03 ± 0·01 and 4·37 ± 0·34 g./g. of wet tissue respectively.

## DISCUSSION

*Content of pyrithiamine in tissues.* Twenty-four hours after the intraperitoneal injection of a single dose (1 mg.) of pyrithiamine, the amount found in the tissues was approximately that of the thiamine usually present in the same tissue, except in the brain where the pyrithiamine content was notably less. If the animals did not receive thiamine (Table 2), the pyrithiamine content decreased in liver, kidney and skeletal muscle, but increased in brain, where, at the end of 12 days, it reached a concentration very near to that of thiamine usually present in normal rats.

The considerable thiamine needs of the brain, an organ with predominantly carbohydrate metabolism, are probably normally met by uptake of thiamine from the liver. When, however, pyrithiamine is present in excess of thiamine in the liver, the brain takes up the pyrithiamine. As a consequence, the content of pyrithiamine in brain greatly increases (Table 2), replacing thiamine in the tissue and inducing the early appearance of neuromuscular signs of beri-beri in the rats. This is a peculiar feature of the pyrithiamine treatment, in contrast with alimentary thiamine deficiency. Under similar experimental conditions, De Caro, Rindi, Perri & Ferrari (1958) found that intraperitoneal injection of pyrithiamine to rats or mice resulted in a marked early decrease of the total thiamine content of the brain, the organ in which we have found the highest pyrithiamine concentration. The present results support the view that pyrithiamine induces a thiamine deficiency almost exclusively in the brain.

The daily administration, by mouth, of a mixture of pyrithiamine and thiamine, in suitable proportions, resulted in a continuous and gradual increase of the pyrithiamine content in all the tissues examined (Table 3). De Caro, Rindi, Perri & Ferrari (1958) showed that under the same conditions there was a decrease of the total thiamine concentration of the tissues, quite comparable with that found after administration of pyrithiamine alone, but requiring a far longer period of treatment before being reached, perhaps as a consequence of the daily intake of thiamine.

*Form of pyrithiamine in the tissues.* The results obtained after treatment of liver extract with phosphatase suggest that, 24 hr. after the intraperitoneal injection of a rat with 1 mg. of pyrithiamine, the liver contains pyrithiamine which is practically all in a phosphorylated form. Pyrithiamine *in vitro* inhibits the hepatic (Eich & Cerecedo, 1954) and intestinal (Cerecedo, Eich & Bresnick, 1954) thiaminokinase. It seems likely that this enzyme could phosphorylate pyrithiamine, especially when present in the amount found in the liver 24 hr. after its injection.

## SUMMARY

1. A microfluorimetric method for the determination of pyrithiamine in animal tissues is described.

2. The intraperitoneal injection of 1 mg. of pyrithiamine in rats, reared on a thiamine-deficient diet, caused an initial increase of the pyrithiamine content in the liver, muscle, kidney and brain, followed by a steady decrease in all the tissues except the brain, where a final increase of up to 200 % was found.

3. The daily oral administration of 210 μg. of pyrithiamine together with 33 μg. of thiamine, for 20 days, induced a gradual increase of the pyrithiamine content in all the tissues examined.

4. After a single intraperitoneal injection of 1 mg., pyrithiamine was found 24 hr. later in the liver almost all in a phosphorylated form.

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# The Chromatography of the Meromyosins on Diethylaminoethylcellulose

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In a previous chromatographic study of rabbit L-myosin (Perry, 1960) the distribution of adenosine-triphosphatase activity in the main eluted fraction of this protein did not appear to be compatible with enzymic homogeneity. Brahms (1959, 1960) has also reported the separation of components of different adenosine-triphosphatase activity from rabbit myosin. The relation of these findings to the subunit structure of myosin is far from clear but they suggested that re-investigation of the distribution of adenosine-triphosphatase activity between the light and heavy meromyosins (Szent-Gyorgyi, 1953; Gergely, 1953; Mihalyi & Szent-Gyorgyi, 1953) might throw some light on this problem. There is accumulating evidence of the heterogeneity of the meromyosins (Lowey & Holtzer, 1959; Fryar & Gibbs, 1960; Szent-Gyorgyi, Cohen & Philpott, 1960) and the solubility properties of heavy meromyosin compared to myosin and to light meromyosin make this protein particularly suitable for chromatographic studies with diethylaminoethylcellulose.

The present paper is concerned with a chromatographic investigation of the standard light and heavy meromyosin preparations and the distribution of adenosine-triphosphatase activity in the fractions which can be isolated from these proteins. Evidence is presented which suggests that fragments, other than heavy meromyosin, which result from tryptic digestion of myosin, possess appreciable adenosine-triphosphatase activity. A preliminary note of some of the findings reported here has appeared earlier (Mueller & Perry, 1960).

## METHODS

*Chromatography.* Diethylaminoethylcellulose was prepared by the method of Peterson & Sober (1956) and the conditions of chromatography were as previously described (Perry, 1960). Commercial diethylaminoethylcellulose (Whatman powder DE 50), manufactured by W. and R. Balston Ltd., was not found satisfactory for separating fractions from heavy meromyosin. Protein solutions were equilibrated against the appropriate buffer by dialysis and centrifuged for 20 min. at 10 000*g* before application to the column, which had previously been fully equilibrated against the buffer. Usually 150–300 mg. of protein was applied in 1·0–1·5% solution to a 2 cm. × 20 cm. column containing about 7 g. of diethylaminoethylcellulose. Elution was carried out at 60 cm. water pressure and a flow rate of 5–10 ml./hr. For gradient elution a 250 ml. conical mixing chamber was used. Elution was followed by measurement of $E_{1\,cm.}$ at 280 and 260 m$\mu$. Estimations of protein recovery were made by measuring the areas of the eluted peaks on the plotted chromatograms. All chromatographic procedures and preparations of protein solutions were carried out at 1–2°.

*Heavy meromyosin.* L-Myosin, prepared as described by Perry (1955), was digested with trypsin at 23° for 10 min. by the method of Szent-Gyorgyi (1953) and immediately dialysed against 10 vol. of 6·7 mM-phosphate Sørensen buffer (pH 7·0). The precipitated light meromyosin was separated by centrifuging; the heavy meromyosin was salted out by $(NH_4)_2SO_4$ in the range 40–55% saturation, dissolved in 0·15 M-KCl–20 mM-2-amino-2-hydroxymethyl-propane-1:3-diol(tris)–HCl (pH 7·6) and stored at 1–2°.

*Light meromyosin.* The digestion, carried out as described for heavy meromyosin, was usually stopped after 12 min. and the digest kept at 1–2° for 10 days (Szent-Gyorgyi, 1953). The light meromyosin was precipitated by dialysis against 10 vol. of 6·7 mM-phosphate Sørensen buffer (pH 7·0), the precipitate washed two or three times with the same buffer and redissolved by addition of solid KCl to bring the concentration to 0·5 M. This process was repeated twice more and the light meromyosin stored in 0·5 M-KCl.

*Enzyme assays.* Adenosine-triphosphatase assays were carried out at 25° and pH 7·6 as described by Perry (1960). Three different concentrations of enzyme from a given fraction were used and specific activities were calculated from the straight-line graphs obtained by plotting phosphate liberated against amount of protein. Specific activities were expressed as $\mu$g. of phosphorus liberated in 5 min. by 1 ml. of protein solution of $E_{1\,cm.}$ 1·0 at 280 m$\mu$; 1 ml. of protein solution of $E_{1\,cm.}$ at 280 m$\mu$ contained, for L-myosin, light meromyosin and heavy meromyosin, 0·272, 0·320 and 0·248 mg. of N respectively.

*Measurements of* pH. Buffers were diluted to the concentration used and their pH was measured at room temperature (about 18°) with the glass electrode (Electronic Instruments Ltd.). The electrode was standardized at pH 6·99 with Soloid standard buffer tablets (Burroughs Wellcome and Co.).

*Materials.* The trypsin used was a twice-recrystallized preparation containing 50% of $MgSO_4$ (supplied by Sigma Chemical Co.). The soya-bean trypsin inhibitor was

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supplied by the Nutritional Biochemicals Corp., Cleveland, Ohio.

*Ultracentrifuging.* Sedimentation studies were carried out at 20° on the Spinco analytical ultracentrifuge, model E. The composition of the solvent was 0·15 M-KCl–20 mM-tris–HCl (pH 7·6) in all experiments.

*Starch-gel electrophoresis.* The procedure was carried out in 22 mM-sodium borate buffer (pH 9·1) according to Smithies (1955), with the modification of Hall (1959). The protein was applied on filter-paper strips, the operating potential was 4–6 v/cm. and electrophoresis was carried out for 3–6 hr. at room temperature (about 18°).

## RESULTS

### *Heavy meromyosin*

Application of the heavy meromyosin preparations purified with $(NH_4)_2SO_4$ to diethylaminoethylcellulose columns equilibrated against 0·15 mM-KCl–20 mM-tris–HCl (pH 7·6 or 8·3) yielded two main fractions (Fig. 1). Under these conditions 11–17 % of the total material applied, estimated on an $E_{280}$ basis, was not held on the column and passed through unretarded (fraction H1). The bulk of the material (fraction H2) was eluted with a peak at 0·20 mM-KCl–20 mM-tris–HCl (pH 7·6 or 8·3) on application of a gradient to the column. This fraction represented 83–89 % of the total material eluted. The peak with a maximum at a chloride concentration of 0·195–0·205 equiv./l. was fairly symmetrical, although a moderate degree of trailing was apparent with gradient elution. Stepwise application of 0·21 M-KCl–20 mM-tris–HCl (pH 7·6) eluted fraction H2 as a sharp peak. Subsequent application of 2 M-KCl–20 mM-tris–HCl (pH 7·6) eluted an insignificant amount of material absorbing at 280 or 260 mμ (less than 1 % of total eluted material).

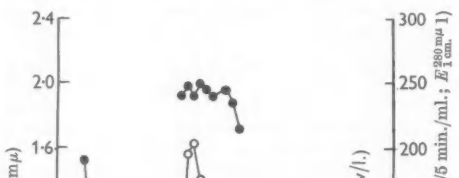


Fig. 1. Chromatography of heavy meromyosin on diethylaminoethylcellulose. Protein solution (25 ml.; $E_{1\,cm.}$ 7·1 at 280 mμ) in 0·15 M-KCl–20 mM-tris–HCl (pH 8·3) was applied to a column 21·5 cm. × 2 cm. diam. Gradient to 0·35 M-KCl–20 mM-tris–HCl at point marked *A* and step to 2·0 M-KCl–20 mM-tris–HCl at *B*. ○, *E* at 280 mμ; ●, specific adenosine-triphosphatase (ATPase) activity; ■, chloride concentration.

Re-chromatography of fraction H2 obtained from the original purified heavy meromyosin preparation by stepwise elution between 0·15 M-KCl and 0·35 M-KCl in 20 mM-tris–HCl (pH 7·6) indicated that a practically complete separation from fraction H1 was obtained (Fig. 2).

*Adenosine-triphosphatase activity.* Study of the specific adenosine-triphosphatase activity of the fractions obtained by chromatographing purified heavy meromyosin revealed that significant activity was associated with both peaks (Fig. 1). The specific activity remained constant throughout the major portion of the H2 peak and although some fall could be detected in the trailing edge, the picture in general was of enzymic homogeneity throughout the bulk of the fraction. The average specific activity of fraction H2 was 240 units. This represented about a 20 % increase on the unchromatographed heavy meromyosin preparations, which were themselves about 50 % more active that the original L-myosin preparations from which they were obtained.

Fraction H1 was much less enzymically homogeneous. The earliest eluted fractions approached the original heavy meromyosin in specific activity but the enzymic activity fell off sharply in a linear fashion in subsequent fractions so that at the $E_{280}$ peak the activity had fallen by more than 50 % (Fig. 1). The average specific activity, determined on the combined fractions of peak H1, was equal to about 40 % of that of fraction H2. The high values of the faster components of the H1 peak varied somewhat, but never reached the values obtained for specific adenosine-triphosphatase activity of the H2 peak.

The enzymic activity of fraction H1 did not appear to be due to gross contamination with material from fraction H2, for its chromatographic characteristics were different and on re-chromatography the fraction H1 was virtually completely eluted when the buffer concentration reached 0·15 M-KCl–20 mM-tris–HCl (pH 7·6) (Fig. 2).

*Ultracentrifuge studies.* Ammonium sulphate-purified heavy meromyosin sedimented in the ultracentrifuge as a sharp peak. If concentrations of the protein were sufficiently high the presence of slower components could be detected by (*a*) the blunting of the angle of the trailing edge, (*b*) slight

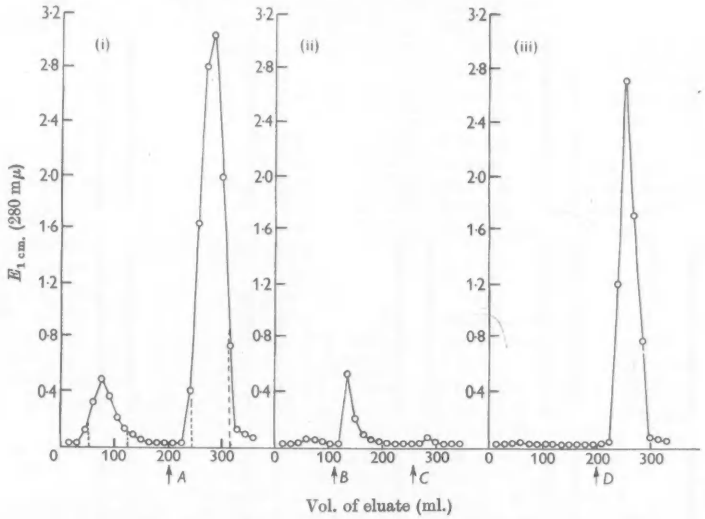


Fig. 2. Re-chromatography of fractions isolated from heavy meromyosin. (i) Heavy meromyosin. Protein solution (50 ml.; $E_{1\,cm.}$ 3·0 at 280 mμ) in 0·15 M-KCl–20 mM-tris–HCl (pH 7·6) was applied to a column 19·5 cm. × 2 cm. diam. Step to 0·35 M-KCl–20 mM-tris–HCl at *A*. Portions of peaks between broken lines were used for re-chromatography. (ii) Re-chromatography of fraction H 1. Protein solution (64 ml.; $E_{1\,cm.}$ 0·198 at 280 mμ) in 20 mM-tris–HCl (pH 7·6) was applied to a column 7·0 cm. × 1·0 cm. diam. Step to 0·15 M-KCl–20 mM-tris–HCl at *B* and step to 0·35 M-KCl–20 mM-tris–HCl at *C*. (iii) Re-chromatography of fraction H 2. Protein solution (44 ml.; $E_{1\,cm.}$ 2·16 at 280 mμ) in 0·15 M-KCl–20 mM-tris–HCl (pH 7·6) was applied to a column 19·0 cm. × 2 cm. diam. Step to 0·35 M-KCl–20 mM-tris–HCl at *D*.

elevation of the base line on the meniscus side and (*c*) the appearance of a small slow peak (Fig. 3*a*). The exact pattern varied somewhat in different preparations. In aged preparations faster peaks, presumably due to aggregation, appeared.

Chromatography on diethylaminoethylcellulose removed the slower components, for even at high concentrations fraction H 2 sedimented as a single peak (Fig. 3*b*). The base line from the peak to the meniscus was essentially straight and there was no blunting of the angle the peak made with the base line. In fraction H 1, concentrated by vacuum dialysis or by precipitation with 90 % $(NH_4)_2SO_4$, at least three components could be recognized on ultracentrifuging (Fig. 3*c*).

Confirmation of the heterogeneity of fraction H 1 was obtained by starch-gel electrophoresis. This fraction moved as three well-defined bands and occasionally a fourth could be recognized. Fraction H 2 showed only slight irregular movement and gave no defined pattern under our conditions of electrophoresis.

*Ammonium sulphate fractionation.* In precipitating sharply between 45 and 50 % of $(NH_4)_2SO_4$, fraction H 2 resembled unchromatographed heavy meromyosin (Fig. 4). The precipitation of the H 1 fraction, however, in general extended over a broader zone of $(NH_4)_2SO_4$ concentrations. Even at 80 % saturation precipitation was not complete (Fig. 4).

*Light meromyosin*

In the region of *I* 0·15 light meromyosin is insoluble at pH 7·6 but satisfactory solutions for chromatography were obtained by increasing the pH to 8·2. In this respect light meromyosin resembled L-myosin (Perry, 1960). When applied in 0·15 M-KCl–20 mM-tris–HCl (pH 8·25) to a column of diethylaminoethylcellulose previously equilibrated against the buffer, light meromyosin was separated into three distinct fractions (Fig. 5). Fraction L 1 passed through the column with the original eluent without apparent retardation and was eluted as a fairly symmetrical peak. Further elution of protein began when the chloride concentration of the buffer was gradually raised by a gradient at 0·185–0·195 equiv./l. The elution of this fraction (fraction L 2) continued over a wide range of rising KCl concentration, leading to a rather broad asymmetric and trailing peak. The maximum occurred at 0·20–0·21 equiv. of chloride/l. but in

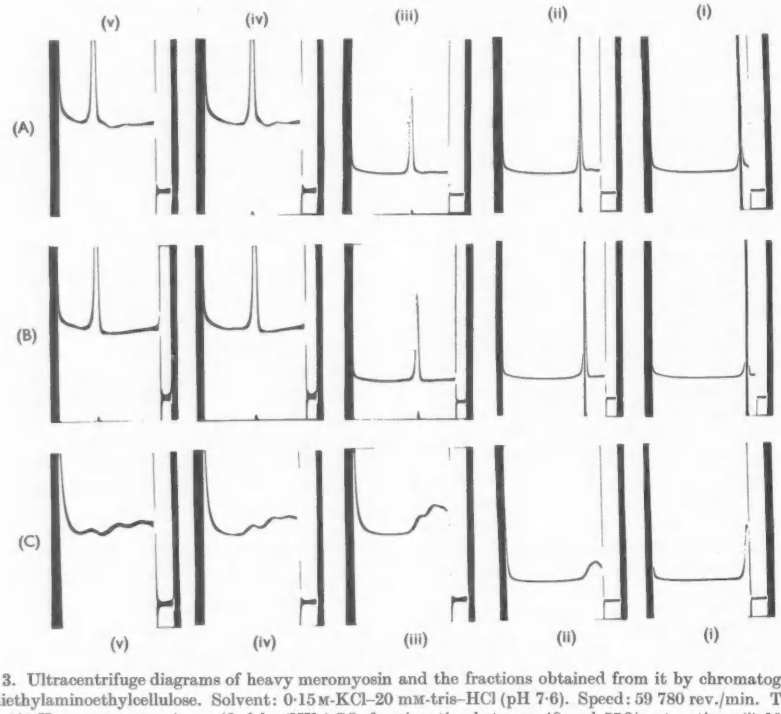


Fig. 3. Ultracentrifuge diagrams of heavy meromyosin and the fractions obtained from it by chromatography on diethylaminoethylcellulose. Solvent: 0·15 M-KCl–20 mM-tris–HCl (pH 7·6). Speed: 59 780 rev./min. Temp.: 20°. (A) Heavy meromyosin purified by $(NH_4)_2SO_4$ fractionation between 40 and 55% saturation: (i) 19 min., 65°; (ii) 35 min., 65°; (iii) 59 min., 65°; (iv) 75 min., 35°; (v) 91 min., 35°. (B) Heavy meromyosin fraction H2. Eluted after removal of the H1 fraction by stepwise raising of the salt concentration from 0·15 M-KCl– to 0·35 M-KCl–20 mM-tris–HCl (pH 7·6): (i) 18 min., 65°; (ii) 34 min., 65°; (iii) 58 min., 65°; (iv) 74 min., 35°; (v) 90 min., 35°. (C) Heavy meromyosin fraction H1. This fraction passed unheld through the diethylaminoethylcellulose column when heavy meromyosin was applied in 0·15 M-KCl–20 mM-tris–HCl (pH 7·6). (i) 9 min., 65°; (ii) 25 min., 65°; (iii) 49 min., 35°; (iv) 73 min., 35°; (v) 97 min., 35°.

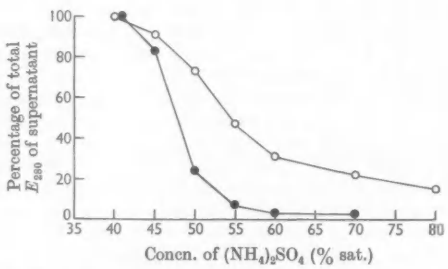


Fig. 4. Solubility of fractions H1 and H2 from heavy meromyosin in $(NH_4)_2SO_4$ solutions. Solid $(NH_4)_2SO_4$ added to about 4 mg. of protein in a final volume of 5 ml. Protein precipitated was estimated by measuring $E_{280}$ of the supernatant after high-speed centrifuging. ○, Fraction H1; ●, fraction H2.

general the peak was much broader than that of the H2 fraction from heavy meromyosin. Re-chromatography of fraction L2 indicated that its behaviour was consistent and that the initial separation effectively removed fractions L1 and L3.

Application of 2 M-KCl–20 mM-tris–HCl (pH 8·2) after fraction L2 had been completely removed from the column eluted a third fraction (fraction L3). This fraction differed from the two preceding fractions in that $E_{260}$ in the ascending portion of the peak rose relative to $E_{280}$ and exceeded it over most of the peak. Fraction L3 was probably similar to, or derived from, the ribonucleoprotein component previously isolated from myosin by chromatography (Perry, 1960). From the ratios of $E$ at 260 and 280 m$\mu$ (Warburg & Christian, 1941) the nucleic acid content of this fraction was in the range 10–20%.

Fractions L1 and L2 were fairly constant in amount, representing on an $E_{280}$ basis 12–15% and 70–80% respectively of the eluted protein. Fraction L3 showed the greatest variation, ranging from 6 to 25%. This was no doubt due to the fact that because of the appreciable trailing of fraction L2 it was sometimes impracticable to wait until the eluate had reached an $E_{280}$ of zero before applying the step to 2M-KCl–20 mM-tris–HCl (pH 8·2).

*Adenosine-triphosphatase activity.* Unchromatographed light meromyosin possessed 15–20% of the specific adenosine-triphosphatase activity of the original L-myosin. This activity could not be removed either by repeated precipitation of the light meromyosin or by increasing the time of the tryptic digestion of the L-myosin from 10 to 12 min.

The distribution of enzymic activity in the fractions separated from light meromyosin is shown in the upper part of Fig. 5, in which the specific adenosine-triphosphatase activity is plotted against eluate volume. Clearly the major portion of the activity was associated with fraction L2, where it showed a characteristic and consistently observed distribution. The specific activity was highest at the front of the peak, rapidly fell in the next few fractions and then remained fairly constant at a level slightly below that of the unchromatographed preparation. In spite of this consistent pattern of distribution, in different preparations the actual values ranged between 100 and 200 units at the front and between 50 and 15 units for the remaining portion of the peak. A barely significant level of adenosine-triphosphatase activity, which was too low to establish a distribution pattern, was observed in fraction L1. Enzymic activity was consistently present in fraction L3, although the activity was lower than that in fraction L2. The pattern of distribution was consistent but the adenosine triphosphatase tended to be higher the higher the proportion of the total eluted material represented by fraction L3. Highest values were obtained in the front of the L3 peak and the activity declined in a fairly linear fashion to the trailing edge.

## DISCUSSION

Although the standard preparations of both light and heavy meromyosin are heterogeneous by chromatographic criteria the major fraction obtained from both proteins behaved on diethylaminoethylcellulose very much like the parent myosin molecule (Perry, 1960), i.e. in each case the major eluted peak is obtained at about $I$ 0·2 in the range pH 7·6–8·2.

As was originally reported by Szent-Gyorgyi (1953), we find relatively low but significant adenosine-triphosphatase activity in the light meromyosin fraction. This activity is largely confined to the principal fraction, L2, which itself appears to be enzymically heterogeneous in so far as the specific activity varies in a marked and characteristic way along the eluted peak. Although any undigested myosin would be expected to be found in this fraction the evidence available suggests that myosin is not responsible for the enzymic activity. The evidence available suggests that it

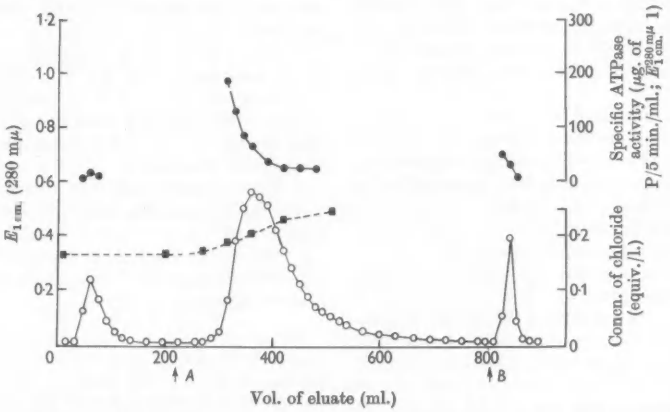


Fig. 5. Chromatography of light meromyosin. Protein solution (17 ml.; $E_{1\,cm.}$ 4·5 at 280 mμ) in 0·15M-KCl–20 mM-tris–HCl (pH 8·3) applied to a column 10·5 cm. × 2 cm. diam. Gradient to 0·35M-KCl–20 mM-tris–HCl was applied at point *A* and step to 2·0M-KCl–20 mM-tris–HCl at *B*. ○, *E* at 280 mμ; ●, specific adenosine-triphosphatase (ATPase) activity; ■, chloride concentration.

may be an oversimplification to assume that light meromyosin is devoid of enzymic activity.

The ribonucleoprotein normally associated with myosin preparations (Mihalyi, Laki & Knoller, 1957; Liubimova & Fain, 1958; Perry & Zydowo, 1959; Perry 1960) is entirely associated with the light meromyosin fragment. Szent-Gyorgyi *et al.* (1960) have recently independently reported a similar association. If this ribonucleoprotein has any biosynthetic function (cf. Perry & Zydowo, 1959) its presence may explain the reported differences in the turnover of light and heavy meromyosins (Velick, 1956; Schapira, Broun, Dreyfus & Kruh, 1956).

On the other hand, the main component fraction H2 isolated from heavy meromyosin by the chromatographic technique presents convincing evidence of a high degree of homogeneity from sedimentation studies and from its behaviour on re-chromatography. Further, the specific adenosine-triphosphatase activity of this component was consistently about 20% higher than that of unchromatographed meromyosin, and a high degree of enzymic homogeneity was indicated by the fact that specific activity remained remarkably constant over most of the eluted peak. A fall in activity in the last few fractions could be due to inactivation of the enzyme, but if the decrease was the consequence of the presence of a less readily eluted contaminant the amount present could not have exceeded a low percentage of the whole. The distribution of adenosine-triphosphatase activity over the eluted peak of fraction H2 is much more compatible with the concept of a homogeneous enzyme preparation than are the results with myosin. The results obtained with the subunit emphasize the significance of those obtained with myosin but as yet do not indicate an explanation of the earlier findings (Perry, 1960; Brahms, 1959, 1960).

The fraction not held by diethylaminoethylcellulose at 0·15M-potassium chloride–20 mM-tris–hydrochloride (pH 7·6 or 8·2), usually representing 10–15% of the heavy meromyosin preparations, is of particular interest. It contains components which are probably responsible for the heterogeneity observed in ultracentrifuge studies by other workers (Lowey & Holtzer, 1959; Fryar & Gibbs, 1960; Mihalyi & Harrington, 1959), and might on general grounds be presumed to be of smaller molecular size than heavy meromyosin itself. This fraction possesses appreciable adenosine-triphosphatase activity, and although the possibility of slight H2 contamination cannot be completely excluded it is unlikely that it accounts for the enzymic activity of this fraction. In view of the sedimentation results, the consistent chromatographic behaviour, the salting-out characteristics and electrophoretic behaviour the evidence is strongly suggestive that there exist in the standard heavy meromyosin preparations protein fragments, other than the main component, which possess adenosine-triphosphatase activity. This conclusion is supported by the results of preliminary experiments which indicate that the H2 component can be degraded still further with trypsin to produce fragments similar to those present in the H1 fraction.

The marked heterogeneity apparent in both light and heavy meromyosin preparations emphasizes the need for caution in interpreting the results of a number of important studies on these components, i.e. end-group analysis (Middlebrook, 1958, 1959), turnover studies (Velick, 1956; Schapira *et al.* 1956) and investigations based on antigenic properties (Marshall, Holtzer, Finck & Pepe, 1959; Szent-Gyorgyi & Holtzer, 1960).

## SUMMARY

1. About 85% of the standard preparations of heavy meromyosin can be shown by chromatography on diethylaminoethylcellulose to consist of a component, fraction H2, which is of high homogeneity by sedimentation, chromatographic and enzymic criteria. The enzyme activity of this fraction was higher than that of the unchromatographed heavy meromyosin and the myosin from which it was derived.

2. The minor fraction separated from heavy meromyosin, fraction H1 has been shown to consist of at least three components by sedimentation studies and starch-gel electrophoresis. Evidence of enzymic heterogeneity in this fraction is also presented.

3. Appreciable adenosine-triphosphatase activity is associated with the H1 fraction and the results strongly suggest that it contains fragments smaller than the H2 component derived either from the latter or from the myosin molecule during the trypsin treatment.

4. Light meromyosin has been separated into three main fractions, one of which is rich in nucleic acid.

5. Small but significant amounts of adenosine-triphosphatase activity were present in all preparations of light meromyosin.

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